The effect of nutritional status on goldfish (*Carassius auratus*) gut microbiota composition and energy homeostasis

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Abstract

The microorganisms within the intestinal tract (termed gut microbiota) have been shown to interact with the gut-brain axis, a bidirectional communication system between the gut and the brain mediated by hormonal, immune, and neural signals. Through these interactions, the microbiota might affect behaviours, including feeding behaviour, digestive/absorptive processes (e.g., by modulating intestinal motility and the intestinal barrier), metabolism, with repercussions on the energy homeostasis and health of the host. To date, research in this field has mostly focused on mammals. Studies on non-mammalian models such as fish may provide novel insights into the specific mechanisms involved in the microbiota-brain-gut axis. The goldfish gut microbiota was initially analyzed in various regions of the gut (foregut, midgut and hindgut), with similarities found among gut regions and fish samples. The effects of two and four weeks of fasting and a synbiotic-based diet on the goldfish midgut microbiota was then analyzed. Fasting resulted in no significant differences in the midgut microbiome diversity in goldfish. There were significant differences between two and four-week fasted groups with regards to the abundance of specific bacterial taxa (species composition). The synbiotic diet induced a decrease in the microbiome biodiversity in comparison to the control diet, and significant changes in the species composition. Goldfish fed the synbiotic diet had a significant increase in food consumption compared to fish fed the control diet. I examined the effects of fasting and a synbiotic diet on the expression of appetite regulators. Fasting had no effects on the mRNA expressions of brain orexin and CART1 or foregut GLP-1, but decreased brain CART2 and foregut CCK, and PYY, which was expected, as these are anorexigenic hormones. The synbiotic diet had no effect on relative

brain mRNA expression of brain orexin, CART1 and CART2 or foregut CCK and GLP-1 but increased mRNA foregut expression for PYY. Overall, this study provides new insights into the effects of nutritional status on the goldfish gut microbiota and energy homeostasis, and suggests that alterations of the gut microbiota due to changes in nutrition may regulate the central and peripheral expression of genes related to appetite and digestion.

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CART1	Cocaine-amphetamine-regulated transcript 1
CART2	Cocaine-amphetamine-regulated transcript 2
CCK	Cholecystokinin
FOS	Fructooligosaccharide
GALT	Gut-associated lymphoid tissue
GH	Growth Hormone
GIT	Gastrointestinal tract
GLP-1	Glucagon-like peptide 1
ICV	Intracerebroventricular
IGF-1	Insulin-growth factor
MOS	Mannanoligosaccharides
NPY	Neuropeptide Y
OTC	Oxytetracycline
OX	Orexin
POMC	Proopiomelanocortin
PYY	Peptide YY
qPCR	Quantitative polymerase chain reaction
RT	Reverse Transcription
SCFA	Short chain fatty acid
scFOS	Short-chain fructooligossaccharides
SMX	Sulfamethoxazole

Chapter 1: Gut microbiota and energy homeostasis in fish

1.1 Introduction and overview

1.1.1 Microbiota/Microbiome

The microbiota can be defined as the collection of microorganisms that occupy a particular environment whereas the term "microbiome" refers to the collection of genomes of the microorganisms within the microbiota (Burokas, Moloney et al. 2015). These microbial communities include commensal, mutualistic, and pathogenic microorganisms (Sandrini, Aldriwesh et al. 2015). Multicellular organisms, including plants and animals live in close association with microorganisms, and harbour such complex microbial communities in and on themselves, from the skin surface to the gastrointestinal tract (GIT) (Sandrini, Aldriwesh et al. 2015). The microbiota may be contracted and developed through exposure to environmental factors. Given the potentially large impact of the microbiota on the host health, an increasing number of studies have been carried out to characterize and determine the mechanisms of action of these microbes.

The diverse microbial community that colonizes the GIT (gut microbiota) plays a critical role in modulating the host's physiology (Rosenbaum, Knight et al. 2015; Tarnecki, Burgos et al. 2017; Feng, Chen et al. 2018). The gut microbiota has lived in symbiotic association with the vertebrate host for millions of years, the host providing a nutrient-rich environment for the microbiota, and the microbiota providing metabolic, protective, and structural functions for the host (Rosenbaum, Knight et al. 2015; Gonçalves and Gallardo-Escárate 2017; Tarnecki, Burgos et al. 2017). The gut microbiota is often considered as an "extra organ", as it plays a key role in the intestinal development and physiology, as well as overall development, growth and health (Feng, Chen et al. 2018). Recent studies suggest that the gut microbiota is involved in energy

homeostasis by regulating feeding, digestive and metabolic processes, as well as the immune response (Burokas, Moloney et al. 2015; Mayer, Tillisch et al. 2015; Gonçalves and Gallardo-Escárate 2017; Johnson and Foster 2018). In particular, the gut microbiota influences the brain-gut axis, the bidirectional communication between the GIT and the brain (Cryan and O'mahony 2011; Vigneri 2014; Sherwin, Rea et al. 2016), by affecting both gut and brain (Bienenstock, Kunze et al. 2015) and thus helps to maintain host homeostasis.

The function of the gut microbiota and the subsequent physiological responses of the host depend on the composition of the microbes that are present in the intestinal tract (Vigneri 2014). There is a wide variation in the composition of fish gut microbiota between species and individuals, but several phyla have been shown to be dominant, including *Proteobacteria*, *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, and *Fusobacteria* (Eichmiller, Hamilton et al. 2016). To date, most of the studies on gut microbiota have focused on mammals, in particular rodents, and in comparison, little is known about the host-microbe interactions in fish (Tarnecki, Burgos et al. 2017). There are several limitations to using mammalian models, including husbandry constraints, and the use of isogenic strains. Owing to their short life cycles and high offspring numbers, and their diversity in genetics, physiology and immunological features, which can be easily manipulated, fish may represent valuable models to study microbiota in vertebrates (Lescak and Milligan-Myhre 2017; Leulier, MacNeil et al. 2017). In addition, studies on fish gut microbiota may help improve welfare of fish and aquaculture practices. However, notable differences exist between mammals and fish with regards to metabolism and energy expenditure (Sandrini, Aldriwesh et al. 2015) and variations in host-microbe interactions and in contributions to maintaining host homeostasis could be expected between fish and mammals.

1.1.2 Physiological roles of gut microbiota

1.1.2.1 Feeding/digestion/metabolism

Studies in mammals show that microorganisms within the GIT are involved in the regulation of appetite/ingestion, digestion, and metabolism (Duca, Swartz et al. 2012; Fetissov 2017; Read and Holmes 2017; Bliss and Whiteside 2018). For example, germ-free mice lacking a gut microbiota are leaner than normal control mice even when consuming more calories (Duca, Swartz et al. 2012). Furthermore, these mice have lower levels of appetite-regulating hormones such as leptin and ghrelin, indicating that the gut microbiota is involved in the regulation of appetite and metabolism. Microbial secretions, including specific metabolites such as short chain fatty acids (SCFAs), indoles, propionate, butyrate and acetate (Zhang and Davies 2016) affect digestive processes and metabolism. The microbiota interacts with GIT neurotransmitters [e.g. serotonin (Yano, Yu et al. 2015), and the catecholamines dopamine, norepinephrine (Asano, Hiramoto et al. 2012)] and thus influence their effects on gastrointestinal (GI) motility, function and hormone release, as well as feeding behaviour (Yang and Chiu 2017; Strandwitz 2018). Conversely, serotonin and catecholamines released from enteric neurons can influence the microbiota present in the gut and alter release of cytokines and bacterial molecules (Mittal, Debs et al. 2017). Therefore, the gut the microbiota has the potential not only to regulate metabolic processes, but also to alter intestinal bacterial composition and the release of bacterial molecules due to the presence of specific metabolites.

Some of these metabolites can act on enterocytes and regulate their intestinal barrier function (Venkatesh, Mukherjee et al. 2014), absorptive capacity [e.g. monosaccharide absorption (Fredrik Backhed and Clay F. Semenkovich 2004)], and nutrient uptake and storage [e.g. altered enzymatic activity in the gut and fat storage (Franchini, Fruciano et al. 2014)], thus influencing

metabolism [e.g. cholesterol metabolism and adipogenesis (Agustí, García-Pardo et al. 2018)]. Furthermore, metabolites from the gut microbiota can modify the secretory activity of enterocytes, thus affecting the production of gut peptides that modulate gut motility and enzyme secretion (Borre, Moloney et al. 2014; Cani and Knauf 2016). For example, SCFAs receptors have been shown to interact with the enteroendocrine L cells containing the gut hormone Peptide YY (PYY) to influence the colonic PYY expression in rats (Tolhurst et al. 2012), and further influence metabolism. These microbial compounds can influence feeding behavior [e.g. (Wostmann, Larkin et al. 1983; Duca, Swartz et al. 2012; Breton, Tennoune et al. 2016)] directly, by entering the circulation and reaching the brain, or indirectly, by either by activating vagal terminals or by modulating the release of appetite regulating gut peptides (e.g. cholecystokinin, CCK; ghrelin, gastrin), which in turn affect the release of central appetite-regulating neuropeptides (e.g. neuropeptide Y, NPY; proopiomelanocortin, POMC) (Breton, Tennoune et al. 2016; Cussotto, Sandhu et al. 2018; Kim and Claire 2018) (Figure 1.1). The exact mechanisms ruling the communication between the gut microbiota and the brain (termed the "microbiota-gut-brain axis") and how changes within the gut microbiota may impact neuropeptide systems in the brain are still unclear (Cani and Knauf 2016). To date, very few studies have been conducted in fish with regards to the influence of microbiota on feeding and metabolism, but they provide clues to some similarities with mammals in this regard.

The influence of the microbiota on food intake has been examined in a few studies correlating feeding rates and changes in microbiota. However, results are inconsistent and difficult to compare, as several studies and several additives are used. For example, zebrafish fed with *Lactobacillus rhamnosus* have reduced appetite compared to control fish (Falcinelli, Rodiles et al. 2016; Falcinelli, Rodiles et al. 2018). However, carp fed a diet supplemented with

fructo-oligosaccharide (FOS) display changes in microbiota composition (increased levels of heterotrophic aerobic bacteria and lactic acid bacteria) but no changes in feeding rates compared to fish fed a control diet (Hoseinifar, Soleimani et al. 2014). This may indicate that the gut microbiota has a host specific and variable effect on feeding behaviour.

The potential effect of the gut microbiome on metabolism has been examined in a few fish species. In grass carp (*Ctenopharyngodon idella*), many biosynthesis and metabolism pathways of carbohydrates, amino acids and lipids change as the composition of microbiota changes (Ni, Yan et al. 2014). In zebrafish, the colonization of the gut by microorganisms promotes epithelial absorption of fatty acids (Semova, Carten et al. 2012) and fish with intact microbiota have increased lipid accumulation in the intestinal epithelium, and increased expression of genes related to lipid metabolism compared to germ-free fish who lack microbiota (Sheng, Ren et al. 2018). In addition, Japanese flounder (*Paralichthys olivaceus*) fed a diet supplemented with *Bacillus clausii* display higher weight gain, feed efficiency and growth performance compared to fish fed control diets (Ye, Wang et al. 2011). The authors suggest this could be attributed to increased food intake and improved nutrient digestibility (Ye, Wang et al. 2011). All these data suggest a strong influence of the microbiota in fish metabolism.

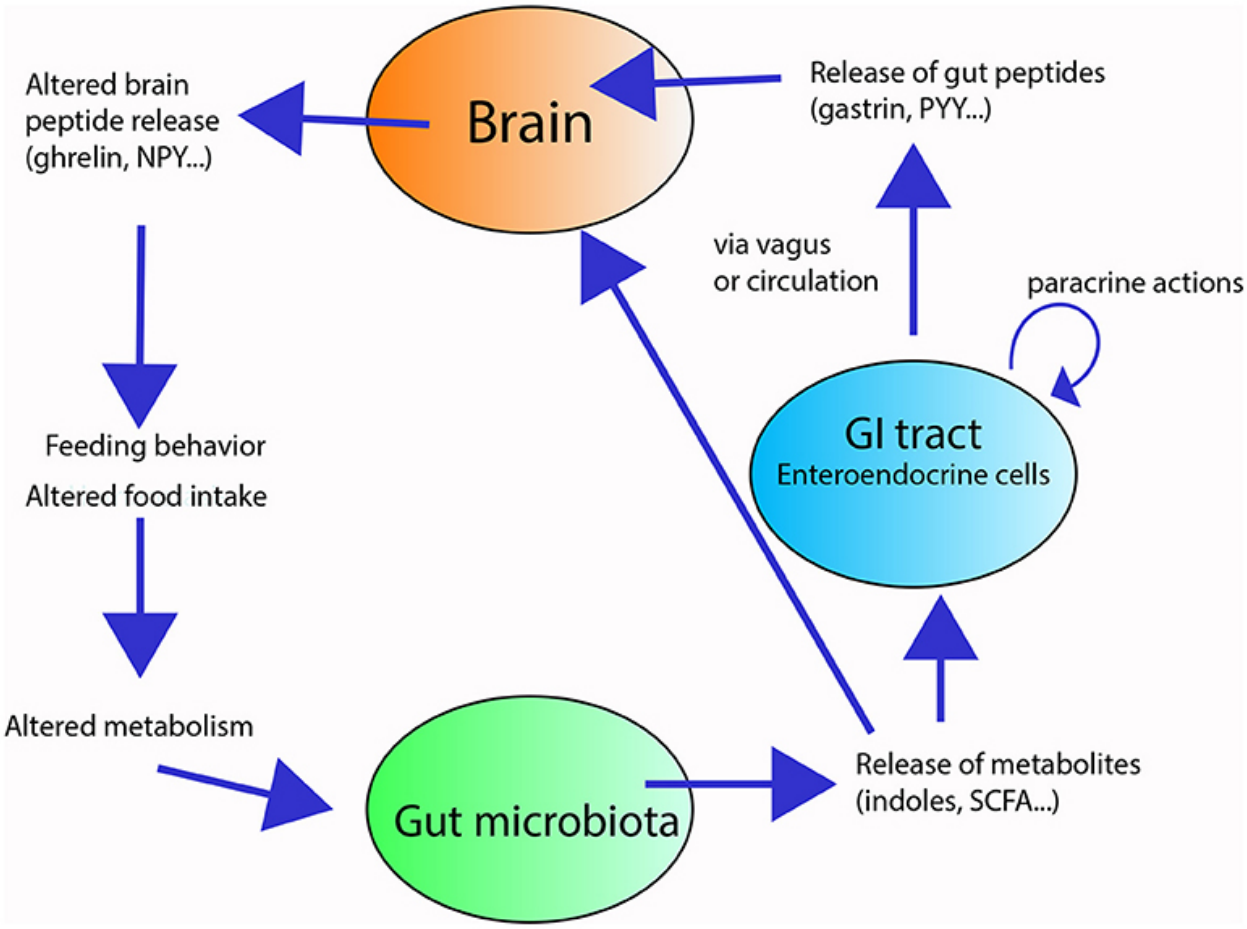


Figure 1.1: Overview of the gut-microbiota-brain axis in feeding and digestion. The gut microbiota releases metabolites in response to substrates present in the gut lumen. These metabolites locally stimulate the enteroendocrine cells of the gastrointestinal tract (GIT) and/or reach the brain. The stimulated enteroendocrine cells release gut peptides, which act locally in the GIT and affect brain feeding centres, altering neuropeptide release and modifying feeding behaviour and energy homeostasis.

1.1.3 Factors affecting fish gut microbiota

Biotic (e.g. genotype, physiological status, pathobiology, life style) and abiotic (e.g. environmental) factors may affect the fish gut microbiota and influence its composition and diversity, as well as its function and metabolic activity, thus affecting feeding, growth, energy storage and health of the fish (Ghanbari, Kneifel et al. 2015) (Figure 1.2). This section will review these intrinsic and extrinsic factors and provide specific examples in which the gut microbiota of various fish has been altered as a result.

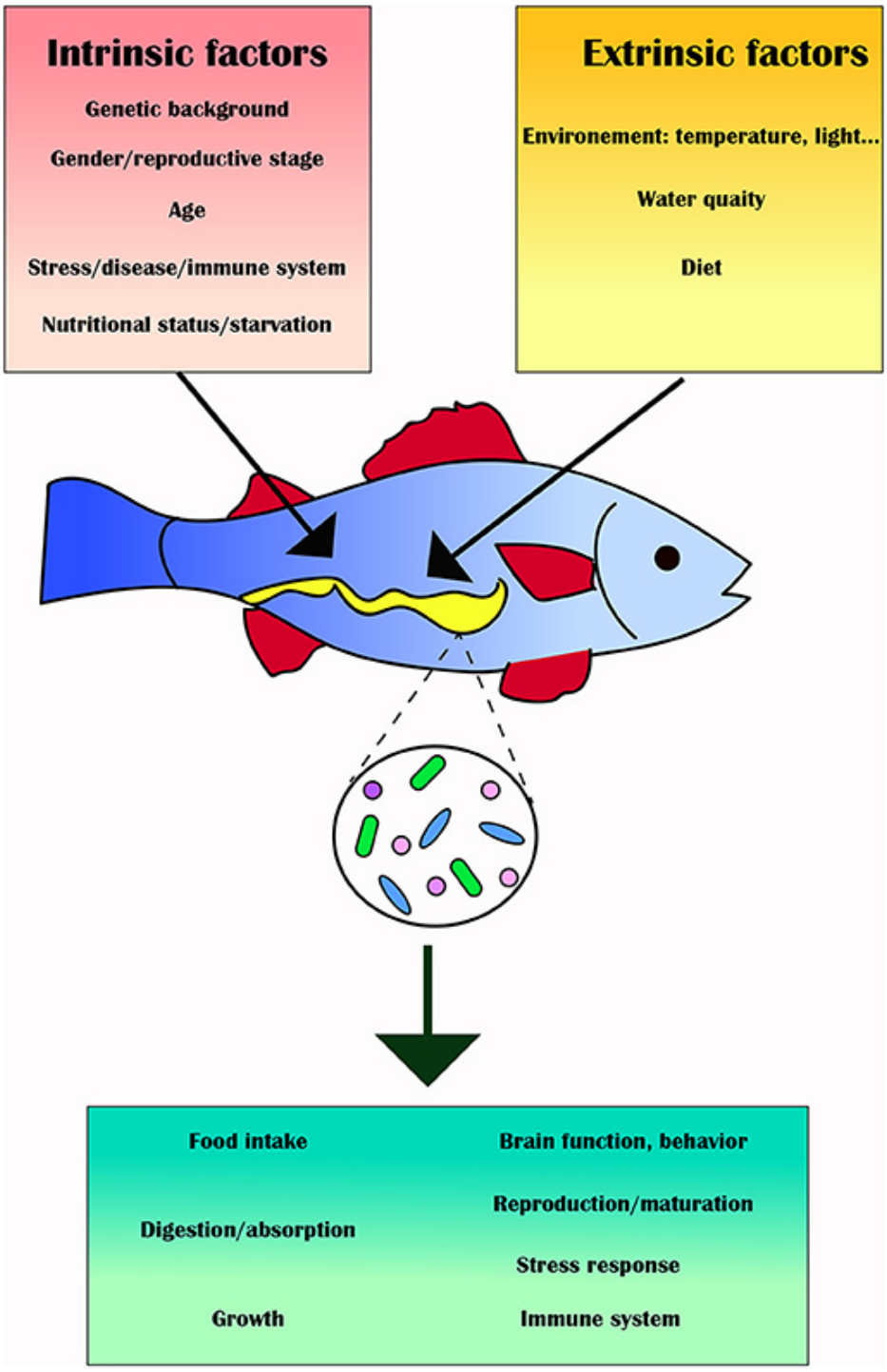


Figure 1.2: Intrinsic and extrinsic factors can alter the gut microbiota and its downstream effects on the fish host.

1.1.3.1 Environmental factors

Initially, fish embryos develop in a relative constant bacteria-free environment (within the egg or the mother). Fish are thus theoretically microbe-free at hatching, and gut microbes acquired post-hatch originate from surrounding environments (Li, Zhou et al. 2017). After hatching, fish are submitted to changing environmental factors (e.g. water composition and quality, and temperature), which can greatly influence the gut microbiota throughout their lifespan (Vatsos 2017). For example, the composition of gut bacteria differs between fish inhabiting freshwater and marine ecosystems (Vatsos 2017). Generally, *Aeromonas* and *Pseudomonas* predominate freshwater fish, whereas in marine fish, *Vibrio* is the most common genus. In black molly (*Poecilia sphenops*), an increase in salinity induces changes in dominant bacterial taxa in the microbiomes (Schmidt, Smith et al. 2015) and in rainbow trout, an increase in temperature results in an increase in microbial growth (Huyben, Sun et al. 2018). Therefore, environmental factors specific to the marine habitat a fish occupies will determine the gut microbiota composition.

The gut microbiota of a species can fluctuate over short time scales such as within one day (Sugita, Fukumoto et al. 1987) and days, or longer time periods (months or years) and this may be the result of seasonal variations (Zhang, Li et al. 2017). Seasonal changes, accompanied by changes in temperature might induce alterations in food consumption due to variations in nutrient loads in the water column (Al-Harbi and Uddin 2004). As a consequence, the composition of the microbiota may be associated with particular seasons. For example, the gut bacterial load of tilapia decreases in winter compared to other seasons and in the gut of hybrid tilapia (*Oreochromis niloticus* X *Oreochromis aureus*), *Pseudomonas*, *Micrococcus*, and *Flavobacterium* are only present in the winter (Al-Harbi and Uddin 2004). Fluctuations in the

fish gut microbiota during the day and throughout seasons may therefore be a result of subsequent changes in diet.

Rearing conditions have been shown to influence the composition of the gut microbiota. For example, in Atlantic salmon, fish held in two different holding conditions (indoor recirculating aquarium facility and cage culture in an open freshwater "loch" environment) have different microorganism compositions within their gut microbiota (Dehler, Secombes et al. 2017). This indicates the effect of habitat on the gut microbiota. In particular, a controlled environment, aquarium facility, in comparison to a variable environment, open freshwater.

Pollutants and toxins present in the environment may influence the fish microbiota. For example, common carp (*Cyprinus carpio*), exposed to waterborne copper (Meng, Li et al. 2018) and zebrafish exposed to polystyrene microparticles (Jin et al., 2018) display disturbances of the intestinal microbiota related to immunity, which increase their susceptibility to pathogens and inflammation (microbiota dysbiosis). Other kinds of environmental chemicals such as pesticides [e.g. (Kan et al., 2015)], heavy metals [e.g. (Giri et al., 2018)] and antibiotics [e.g. (Zhou et al., 2018a)], can induce gut microbiota dysbiosis associated with changes in the intestinal mucus layer and inflammation in fish, thereby reducing the ability of absorb nutrients.

1.1.3.2 Host-specific intrinsic factors

The variations in microbiota composition among fish might be due to several factors, which are host specific. These factors include phylogeny, genetics and sex, age/life stages, and diet/feeding habits (Egerton, Culloty et al. 2018). Variations in the gut microbiota composition of individual fish from the same species for example may indicate the influence of these host-specific intrinsic factors. Furthermore, alterations in hormones, metabolic processes and other

physiological processes can be predicated to result in the differences present among individual fish gut microbiota communities.

1.1.3.2.1 Genetics/sex

Genetic background influences the gut microbiota and intra- and inter-specific variations in microbiota have been demonstrated. Interspecies differences in the composition of gut microbiota among individuals of the same species may be present. For example, in rainbow trout, some bacterial groups are associated with specific families, perhaps due to different habitats or different diets (Navarrete, Magne et al. 2012). Inter-specific differences in bacterial community structure are seen, even if species exposed to the same environment [e.g. four freshwater larvae of silver carp, grass carp, bighead carp, and blunt snout bream (Li, Yu et al. 2012)]. This indicates the genetic background of these fish is perhaps more influential on the gut microbiota than the standard environment.

The sex of the fish may influence the gut microbiota through sex-specific host-microbe interactions, diet preferences or immune responses (Bolnick, Snowberg et al. 2014). For example, differences in the gut microbiota between sexes of the threespine stickleback (*Gasterosteus aculeatus*) and Eurasian perch (*Perca fluviatilis*) have been reported (Bolnick, Snowberg et al. 2014), but the gut microbiota of the zebrafish is similar between sexes (Liu, Yao et al. 2016; Stephens, Burns et al. 2016). However, given the small number of published reports and the wide variation in studies, the underlying mechanisms are not yet understood.

There are still questions on whether genetics or the environment has greater influence on the gut microbiota. To date, host genetics has been considered the most influential in shaping the fish gut microbiota. Channel catfish (*Ictalurus punctatusand*) and blue catfish (*I. furcatus*) raised

under constant environmental and husbandry conditions have similar gut microbiota compositions, suggesting that a shared environment can overcome differences in host genetics (Bledsoe, Waldbieser et al. 2018). However, studies such as these are variable and no general conclusions can be made.

1.1.3.2.2 Age/sexual maturity

Differences in the microbiota composition have been identified between juvenile and sexually mature individuals (Cantas, Sørby et al. 2012; Vatsos 2017). Zebrafish juveniles have higher bacterial richness in their gut microbiotas than elderly adult fish (Cantas, Sørby et al. 2012; Yan, Li et al. 2016), suggesting an increase response of gut microbiota to higher levels of circulating sex hormone levels in adult compared to juvenile fish (Cantas, Sørby et al. 2012). Furthermore, the gut-associated lymphoid tissue (GALT) may interact differently with the gut microbiota in juvenile and mature zebrafish as this system is not fully developed in the juveniles (Cantas, Sørby et al. 2012). Similarly, in southern catfish, gut microbial diversity increases as the host ages (Zhang, Li et al. 2018). These results suggest a reduction in bacterial richness and bacterial diversity as fish age.

Changes in the gut microbiota have been shown during the early life development stages of fish. In Atlantic salmon (*Salmo salar*), intestinal microbiota compositions vary between embryonic stages, with embryonic communities having lower richness and diversity compared to those of hatchlings (Lokesh, Kiron et al. 2019). In Malaysian Mahseer (*Tor tambroides*), larval, juvenile, and adult stages have higher gut microbiota diversity than fingerling and yearling stages (Mohd Nosi, Syed Jamil Fadaak et al. 2018). This may highlight the plasticity of the gut microbiota during these early life stages in fish.

It is possible that modifications in diet contribute to the differences between juvenile and

mature fish gut microbiota. Fish of different ages and sexual stages might have different nutrient requirements and might adjust their diets and feeding rates to obtain adequate energy intake [e.g. in Gibel carp, juvenile fish require more proteins than pre-adults (Ye, Han et al. 2017)]. As the diet and gut microbiota composition change with age, is likely that the nature of the contribution of the gut microbiota to host homeostasis changes. However, further research is needed to explain this potential relationship between fish age, fish gut microbiota and host energy homeostasis.

1.1.3.2.3 Feeding habits/diet

Feeding habits can greatly influence the structure and composition of the gut microbiota (Vatsos 2017). Gut bacterial diversity is generally lower in carnivores, and progressively increases in omnivores and herbivores (Wang, Ran et al. 2018). For example, the most abundant bacteria in herbivorous fish include *Clostridium*, *Citrobacter* and *Leptotrichia*, *Cetobacterium* and *Halomonas*, in omnivorous fish *Clostridium*, *Halomonas*, and *Cetobacterium*, and in carnivorous fish *Clostridium*, *Cetobacterium* and *Halomonas* (Li, Ni et al. 2014; Liu, Guo et al. 2016; Kashinskaya, Simonov et al. 2018). This trend has been found in both marine and freshwater fish, suggesting that the trophic level is likely one of the most influential factor affecting the gut microbiota composition (Egerton, Culloty et al. 2018). The gut microbiota is therefore shaped by the ability of bacteria to exploit the available nutrients provided in the diet.

The gut microbiota can vary within species of the same trophic level. For example, the gut microbiota of four herbivorous Asian carp species (silver carp, *Hypophthalmichthys molitrix*; bighead carp, *Hypophthalmichthys nobilis*; grass carp, *Ctenopharyngodon idella*; and common carp, *Cyprinus carpio*) reared in the same environmental conditions exhibits inter-specific differences, in particular with regards to the relative abundance of the cellulose

degrading phyla *Firmicutes*, most probably due to species-specific diets (Li, Yu et al. 2018). Differences in cellulose intake is likely to have cause these results, and indicate the effect of trophic level on the gut microbiota composition cannot be generalized among species.

A limited number of studies show that modifying the diet of the fish can result in alterations of the gut microbiota, but this is not always the case. Diets containing guar gum, (non-starch polysaccharide) fed to the omnivorous mullet, *Mugil liza* (Ramos, Romano et al. 2015) or soy proteins to the carnivorous rainbow trout (Bruce, Neiger et al. 2018) induce alterations in the bacterial quantity and composition in the GIT. In zebrafish, administration of dietary nucleotides results in modifications of the microbiota and reduction in fatty acid oxidation in muscle and liver as well as lower inflammatory tone (Guo, Ran et al. 2017). However, in channel catfish, different diets with different protein sources including animal and plant meals only have minimal effects on gut microbiota (Schroeter, Peterson et al. 2018). Therefore, the effect of diet of the fish gut microbiota may be highly individualized and diet specifc.

Extreme dietary changes, such as fasting shape the fish gut microbiota. During times of fasting, morphological changes in the GIT occur due to the reduced nutrient uptake, which may account for changes observed in the gut microbiota (Bruce, Neiger et al. 2018). Furthermore, a depletion of nutrients induces changes in gut microbiota composition to favour bacterial species/communities that use more diverse energy sources and are capable of survival under limited nutrient conditions (Vatsos 2017). Microbiota gut diversity and richness are usually higher under feeding conditions than under fasting conditions as seen in zebrafish (Carnevali, Avella et al. 2013) and in leopard coral grouper (*Plectropomus leopardus*) (Mekuchi, Asakura et al. 2018). In grouper, the dominant phyla are *Proteobacteria* in fasting and

Firmicutes in fed conditions (Mekuchi, Asakura et al. 2018) and in the Asian seabass, *Lates calcarifer*, fasting induces a significant enrichment of *Bacteroidetes* and a depletion of Betaproteobacteria (Xia, Lin et al. 2014).Therefore, the bacterial community structure is highly variable depending on available nutrients.

The act of feeding itself can influence the microbiota. In rainbow trout, a short time after feeding (3 hours) is not sufficient to cause any significant changes in the bacterial composition, but can cause changes in species richness and relative abundance (Mente, Nikouli et al. 2018). Similarly, in Southern catfish, although the diversity remains the same, the relative abundance of bacterial phyla differs at 3, 12, and 24 hours after feeding, suggesting that short term food digestion may alter community structure, but has less effect on the microbial composition (Zhang, Li et al. 2018). Diet and feeding behaviour may therefore be suggested to cause temporal changes in bacterial abundance and diversity.

1.1.4 Gut microbiota manipulation and applications

The role of the fish gut microbiota in host physiology has become increasingly evident with growing research in this field. Several experimental methods have been used to assess the role of the fish gut microbiota via the manipulation of these communities, including gnotobiotic, antibiotic, probiotic and prebiotic, and symbiotic studies (Table 1.1).

Table 1.1: Fish gut microbiota via the manipulation techniques used in research (see references in text).

Microbiota Manipulation	Objective	Potential use	Advantages	Disadvantages	Examples of Host species examined	Representative references
Gnotobiotic	Establish a germfree (gut microbiota absent) host or a known microbiota compositio n (predefined microbiota)	Examine the effects of an absence of microbiota (germ free) on the host physiology or the effects of specific gut microorganisms and/or a predefine microbiota on the host	Control over multiple variables and analysis of host responses to specific changes in microbiota	Complex procedures required to produce and maintain gnotobiotes	Zebrafish	(52), (14)
Antibiotic	Inhibit or eliminate targeted gut microbiota bacteria and or bacterial pathogens	Bacterial disease prevention/ treatment	Bacterial disease prevention/ treatment	Potential to disrupt microbial communities and increase disease susceptibility and bioaccumulation	Zebrafish, Mosquitofish , Black molly	(107), (108), (109)
Probiotic	Establish beneficial gut microbiota bacteria	Use in aquaculture, improve fish health (digestion/ growth), health management, disease prevention	Enhancement of immune function of the host, resistance to pathogens, and overall health	No known disadvantages. Probiotics are considered safe overall.	Zebrafish, Rainbow trout, Malaysia masheer	(113), (114), (115) (116)
Prebiotic	Simulate growth of beneficial gut bacteria	Use in aquaculture, improve fish health (digestion /growth), health management, disease prevention	Enhancement of immune function of the host, resistance to pathogens, and overall health	No known disadvantages. Prebiotics are considered safe overall.	Rainbow trout, Nile tilapia, Common carp fry	(113), (114), (115) (116)
Synbiotic	Create a synergistic effect of probiotic and prebiotic	Use in aquaculture, improve fish health (digestion /growth), health management, disease prevention	Improvement of immune response (better than probiotics alone) and increase growth and feed utilization in host	No known disadvantages. Synbiotics are considered safe overall.	Nile tilapia	(117)

1.1.4.1 Gnotobiotic fish

Gnotobiotic animals (or gnotobiotes) are animals with a known microbiota composition. These include germ-free (or axenic) animals and axenic animals that have been inoculated with known microorganisms. Studies involving gnotobiotic fish allow the control over many variables that affect the development of the microbiota and analysis of host responses to specific gut microorganisms (Lescak and Milligan-Myhre 2017; Vatsos 2017). The disadvantages of this type of study is the complex procedures involved in the production and maintenance of gnotobiotes (Vatsos 2017). Regardless, germ free studies have made significant advancements in the understanding of the gut microbiota.

1.1.4.2 Antibiotics

Antibiotics (or anti-bacterials) can be considered environmental factors affecting the gut microbiota. In the aquatic environment, they may be found naturally or as pollutants discharged as metabolites [such as sulfamethoxazole (SMX) and oxytetracycline (OTC)] through feces or urine of treated humans or animals (Zhou, Limbu et al. 2018). Mosquitofish (*Gambusia affinis*) exposed to antibiotics display lower community diversity and taxonomic composition from both skin and gut microbiomes, compared to untreated fish (Carlson, Leonard et al. 2017) and in zebrafish, exposure to OTC results in a disruption of the intestinal microbiota (Zhou, Limbu et al. 2018). Antibiotics can be used to manipulate the microbiota, as they kill or inhibit the growth of specific bacteria. The administration of antibiotics does not completely eliminate gut microbiota communities but can cause significant changes in the microbial composition.

The use of antibiotics in aquaculture for disease prevention and treatment is common. However, antibiotics may disrupt the microbial communities and increase disease susceptibility

(Hassaan, Soltan et al. 2014; Carnevali, Maradonna et al. 2017). Furthermore, antibiotics can bioaccumulate in animal tissues (Carnevali, Maradonna et al. 2017) and lead the development of drug-resistant bacteria, which can be passed along the food chain (Banerjee and Ray 2017; Fu, Yang et al. 2017). Due to the growing awareness of the disadvantages of antibiotics, strict regulations have been established in the aquaculture industry and alternative methods are being developed and tested (Defoirdt, Sorgeloos et al. 2011). Treatment of fish with beneficial microorganisms (probiotics) is a promising solution to antibiotics, as these probiotics inhibit the colonization of potential pathogens by producing antibacterial peptides and competing for nutrients with detrimental bacteria (Gatesoupe 1999). Probiotics may thus reverse the negative effects of antibiotics and improve fish health. For example, in black molly *Poecilia sphenops*, successful colonization of two probiotic species (*Phaeobacter inhibens* and *Bacillus pumilus*) reverses the negative impacts of antibiotics, and decreases mortality rates (Schmidt, Gomez-Chiarri et al. 2017). Therefore, although antibiotics has been widely used for disease prevention in fish, the disadvantages associated with administering these anti-bacterials has prompted the search for alternative therapies.

1.1.4.3. Pro-, pre-, and symbiotics

Probiotics are live microbial cells or components of microbial cells, which confer a health benefit to the host through promoting beneficial intestinal bacterial species, whereas prebiotics are non-digestible food ingredients that selectively stimulate the growth of probiotics (Ghanbari, Kneifel et al. 2015). The supplementation of probiotics and/or prebiotics into the diet of fish is believed to result in beneficial alterations of the gut microbiota and subsequent changes in metabolism and energy expenditure that are beneficial for the host (Llewellyn, Boutin et al. 2014; Banerjee and Ray 2017; Haygood and Jha 2018). The administration of these supplements

enhance immune function of the host and increase its resistance to pathogens, enhancing general health and indirectly favouring feeding and growth (Topic Popovic, Strunjak-Perovic et al. 2017). These effects highlight the potential for probiotics/prebiotics to enhance fish health by manipulating the fish gut microbiota.

Commonly used probiotics in aquaculture include members of the *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Enterococcus*, *Carnobacterium*, *Shewanella*, *Bacillus*, *Aeromonas*, *Vibrio*, *Enterobacter*, *Pseudomonas*, *Clostridium*, and *Saccharomyces* genera (Carnevali, Maradonna et al. 2017). Multispecies probiotics may be more effective than single-strain probiotics as different strains present in multispecies probiotics increase the chance of survival in the gut [as seen in rainbow trout (Ramos, Weber et al. 2013)]. Probiotics increase the number of beneficial gut bacteria. For example, feeding fish with probiotics results in a higher abundance of core gut bacteria in zebrafish (Siriyappagouder, Galindo-Villegas et al. 2018), and an increase in bacterial diversity in rainbow trout (Ramos, Weber et al. 2013) and Malaysian mahseer (Asaduzzaman, Sofia et al. 2018). Beneficial gut bacteria contribute to the maintaining immune and metabolic homeostasis and protecting against pathogens.

Although exceptions exist, probiotics generally promote feed efficiency and growth in fish [e.g. tambaqui *Colossoma macropomus* (Dias, Abe et al. 2018), Japanese flounder (Ye, Wang et al. 2011), tilapia (Haygood and Jha 2018), carp (Yu, Wang et al. 2018), red seabream *Pagrus major* (Zaineldin, Hegazi et al. 2018), trout (Ramos, Weber et al. 2013)], likely by increasing nutrient absorption and perhaps feeding. An increase in absorption results from changes in intestinal morphology, with higher absorptive surface areas and higher microvilli densities in the intestine [e.g. tilapia (Standen, Rodiles et al. 2015), zebrafish (Falcinelli, Rodiles et al. 2016), Malaysian mahseer (Asaduzzaman, Sofia et al. 2018)]. The effects on growth might

be mediated by changes in the expression of growth related genes. For example, following probiotics treatment, growth hormone (GH) expression levels increase in pituitary of Malaysian mahseer and in liver of yellow perch (*Perca flavescens*) (Shaheen and Eissa 2014), and insulin-growth factor (IGF-1) expression is upregulated in the liver of Malaysian mahseer and yellow perch and in body of European seabass (Carnevali, de Vivo et al. 2006). The increase in growth hormones is accompanied by an increase in body weight in these fish.

Overall, the effects of probiotics on feeding have been little examined to date and remain unclear. In tambaqui (de Azevedo, Fosse Filho et al. 2016), fish fed probiotics (*Bacillus subtilis*) and control diets have similar feeding ratios, and probiotics induce a reduction in appetite in zebrafish [*Lactobacillus rhamnosus* (Falcinelli, Rodiles et al. 2016)] and an increase in food consumption in pacu [*B. subtilis* (Cerozi 2012)]. The discrepancies in results are likely due to variations in the nature and doses of probiotics used. These alterations in feeding might be partially due to the modulation of the expression of genes related to appetite. In larval zebrafish, administration of the probiotic *Lactobacillus rhamnosus* induces a decrease in brain NPY expression and an increase in adipose tissue leptin expression (Falcinelli, Rodiles et al. 2016), and in goldfish, ghrelin intestinal expression is down-regulated in *Lactobacillus acidophilus* fed fish compared those of fed control diets (Hosseini, Miandare et al. 2017). The influence probiotics has on appetite regulation can therefore be suggested to be variable among fish species.

In comparison to probiotics, prebiotics have received less attention with regards to their potential for aquaculture. However, overall, studies show that prebiotics have beneficial effects on growth performance, digestive enzyme activity, as well as disease and stress resistance of the

host (Dawood and Koshio 2016). Common prebiotics used in fish include inulin, fructooligosaccharides (FOS), short-chain fructooligosaccharides (scFOS), and mannanoligosaccharides (MOS) (Ringø, Olsen et al. 2010). Prebiotics have been shown to increase growth performance and feed utilization in some fish [e.g. rainbow trout, (Staykov, Spring et al. 2007; Khodadadi, Abbasi et al. 2018), pacu *Piaractus mesopotamicus* (Hisano, Soares et al. 2018), Nile tilapia (Selim and Reda 2015), Caspian roach (*Rutilus rutilus*) (Soleimani, Hoseinifar et al. 2012), but this is not always the case. For example, growth performance is not affected in common carp fry (*Cyprinus carpio*) fed inulin (Eshaghzadeh, Hoseinifar et al. 2015) or in pacu fed β-glucan (Cerozi 2012). The increase in growth seen in some studies might be in part due to modifications in the intestinal structure (i.e. increases in intestinal villi height and digestive enzyme activities), as seen following probiotics administration. An improved immune system function [e.g. rainbow trout (Staykov, Spring et al. 2007), Caspian roach (Soleimani, Hoseinifar et al. 2012)] and improved stress response [zebrafish (Forsatkar, Nematollahi et al. 2017)] most probably largely contribute to a better growth performance of the fish.

Prebiotics and probiotics may be administered in combinations, known as synbiotics, (Nayak 2010; Huynh, Shiu et al. 2017). Studies show that synbiotics improve the survival and implantation and metabolism of probiotic health-promoting bacteria in the GIT (Cerezuela, Meseguer et al. 2011). Synbiotics have been shown to increase growth performance and feed utilization in the host, which may be a result of providing the host with energy and nutrients, and/or enhanced digestion processes (Ringø and Song 2016). For example, following the supplementation of a probiotic (*Bacillus licheniformis*) and a prebiotic (yeast extract), growth performance is increased in Nile tilapia (*Oreochromis niloticus*), and this is accompanied by an

increase in feed intake and feed utilization (Hassaan, Soltan et al. 2014). The combination of probiotics and prebiotics in the diet results in better immune responses than probiotics alone [e.g. rainbow trout (Rodriguez-Estrada, Satoh et al. 2013)]. This may be the result of the synbiotic having an increased likelihood to survive and colonize the gut and ultimately trigger an immune response.

1.1.5 Conclusions

Several studies strongly suggest that the fish gut microbiota influences the overall health of the host fish with regards to overall physiology, and digestion. Relatively few studies on the effects of the microbiota on energy homeostasis have been conducted to date and large variations exist between results, making them difficult to compare.

First, given the possible influence of genetics and the environment, and the low number of species examined, many more species need to be examined before conclusions can be made. Second, a variety of methods has been used for studying the fish gut microbiota, and the results obtained may vary depending on the experimental methods used, highlighting the need to develop appropriate standardized methods to describe fish microbiota (Vatsos 2017). Studying the influence the gut microbiota may have on fish energy balance is challenging, as several different mechanisms of action are responsible, involving both local and endocrine pathways, different physiological systems (e.g. stress, immunity) and molecules (e.g. hormones, metabolites), and that all these systems interact (e.g. gut-microbiota-brain axis communication). Furthermore, each microorganism within the microbiota might have different actions. In addition, compared to terrestrial animals, fish are more exposed to constant environmental changes that could affect the microbiota.

Manipulating the gut microbiota of fish has great potential for aquaculture use to improve

growth. However promising, the future of probiotics/prebiotics faces several challenges, including appropriate modes of treatment (oral, or in the water) and doses, the characterization of mechanisms of action of individual probiotic organisms, and quality control and regulation (Quigley and Shanahan 2014; Jahangiri and Esteban 2018). The fish model can be useful to understand the gut microbiota in other vertebrate species such as humans. Zebrafish (Carnevali, Avella et al. 2013) and threespine stickleback (Lescak and Milligan-Myhre 2017) have been widely used as they are small fish that can be easily maintained in laboratory conditions, and have rapid development and generation times. In addition, their genomes are readily available and display structural and functional genetic similarities to humans.

Therefore, although progress has been made, much remains to be resolved using fish models for gut microbiota. Nonetheless, the research conducted to date has offered great insights into the mechanisms by which these communities are able to regulate the fish host, and provided insights into improving aquaculture practices, and better understanding the host microbe relationships among other vertebrates including humans, and the development of potential pathological treatments.

The objectives of my study were to 1) analyze the composition of the goldfish (*Carassius auratus*) gut microbiota in various regions of the gut; foregut, midgut, and hindgut, 2) determine the effects of a synbiotic administered diet on the gut microbiota and feeding behaviour, 3) determine the effects of fasting on the gut microbiota, 4) assess the expression of genes related to appetite and digestion in fish fed a control diet, fish fed a synbiotic diet, and fasted fish. This research is important, as there are limited studies and knowledge on gut microbiota and the relation of the gut microbiota to appetite/digestion regulation in fish including goldfish. Fish are the largest and most diverse vertebrate group, and used widely for aquaculture and research

purposes. This study provides further insights into the relationship between the gut microbiota and energy homeostasis in fish.

Co-authorship Statement

A version of this manuscript has been published in the Frontiers of Endocrinology on January 24th, 2019. The roles of the authors were as follows: Robyn Lisa Butt had primary responsibility for this article, prepared the manuscript and made appropriate revisions after peer review. Dr. Helene Volkoff helped in the preparation and revision of the manuscript. All authors listed have made substantial, direct and intellectual contribution to the work, and approved it for publication.

Chapter 2: Goldfish (*Carassius auratus*) gut microbiota composition and the expression of genes related to appetite and digestion

2.1 Abstract

The gut microbiota refers to the large and diverse microbial community in the gastrointestinal (GI) tract. The communication between gut and brain is made in part through the meal-induced secretion of gut peptides, such as cholecystokinin (CCK), which send signals to the brain through either neural or endocrine mechanisms. The gut microbiota can thus influence this gut–brain communication via alterations in the absorptive and secretory capacity of the intestinal epithelial cells. The current knowledge on the fish gut microbiota and how it influences the overall health and metabolic processes in fish is limited. In order to contribute to this area of research, we analyzed the gut microbiota in various gut regions (foregut, midgut, and hindgut) of the goldfish (*Carassius auratus*). Phylum Firmicutes was predominant in all gut regions for all fish samples followed by phylum Proteobacteria and Fusobacteria. No differences in the microbial community were found within similar gut regions of different fish samples, nor between gut regions within the same fish sample. To examine the effects of nutritional status on the gut microbiota of the goldfish, fish were subjected to control or synbiotic diets, as well as fasting. The biodiversity, but not beta diversity or community composition, of the control fish differed from that of synbiotic-fed fish. Fasting did not affect foregut microbiome diversity. The beta diversity of the two-week fasted fish differed from that of the four-week fasted fish. Fasted fish had significant differences between two and four-week fasted groups with regards to bacterial taxa. Fish fed the synbiotic diet had higher food consumption than control fish. I assessed how nutritional status affected the relative brain and gut expression levels of orexigenic (i.e. orexin) and anorexigenic hormones (i.e. cocaine and amphetamine regulated transcript-CART, cholecystokinin-CCK, glucagon-like

peptide 1-GLP-1, peptide YY-PYY). The synbiotic diet did not affect relative brain mRNA expression of orexin, CART1 and CART2 or foregut mRNA expressions of CCK, and GLP-1. However, PYY foregut mRNA expression was higher in the synbiotic fed fish compared to control fish. Fasting did not affect either brain orexin and CART1 mRNA expressions or GLP-1 foregut expression but decreased brain CART2 and foregut CCK and PYY relative mRNA expressions, which is consistent with the anorexigenic actions of these peptides. Overall, the results of this study suggest that alterations of the gut microbiota, through fasting or the administration of synbiotics, might contribute to the regulation of central and peripheral expression of genes related to appetite and digestion in goldfish.

2.2 Introduction

Goldfish (*Carassius auratus*) are freshwater fish belonging to the family Cyprinidae order Cypriniformes. They are native to East Asia, and one of the most commonly kept aquarium fish. Goldfish are easily maintained, and have been widely used as models for endocrinology studies, in particular with regards to feeding behaviour and the expression of genes related to appetite (Blanco, Sundarrajan et al. 2018). A limited number of studies have examined the gut microbiota of goldfish.

The regulation of feeding and energy homeostasis in fish is a complex process involving numerous physiological factors. The gastrotintestinal (GI) tract communicates important information regarding the size and composition of a meal to the brain. Gut peptides secreted by enteroendocrine cells after ingestion of a meal [e.g. cholecystokinin (CCK), glucagon-like peptide 1 (GLP-1), peptide YY (PYY)] send signals to the brain through neural or endocrine mechanisms (Bauer, Hamr et al. 2016). The hypothalamus plays a crucial role in integrating these peripheral signals and produces and secretes hormones involved in appetite regulation [which may be described as appetite stimulators (orexigenic factors) such as orexin or inhibitors (anorexigenic factors) such as cocaine-and amphetamine regulated transcript (CART) (Volkoff 2014)] in order to adjust feeding behaviour according to the body's needs.

Orexin is a neuropeptide which consists of two forms, orexin A and orexin B, which preferably bind to orexin1, and orexin2 receptors receptively (Rønnestad, Gomes et al. 2017). These orexin forms and receptors have been identified in goldfish (Hoskins, Xu et al. 2008). In goldfish, orexin treatment has been shown to increase feeding and locomotive behaviours (Volkoff, Bjorklund et al. 1999) and orexin brain mRNA expression increases following fasting (Abbott and Volkoff 2011), suggesting an orexigenic role for orexin in this species.

CART was originally isolated from rat brain (Douglass, McKinzie et al. 1995), and subsequently described in birds and in some fish species (Rønnestad, Gomes et al. 2017). In goldfish, two forms of CART precursors exist, CART1 and CART2 (Volkoff and Peter 2001). In goldfish, CART injections inhibit food intake (Volkoff and Peter 2000) and fasting results in a decrease in brain mRNA expression of both CART1 and CART2 (Volkoff and Peter 2001), indicating an anorexigenic role for CART in goldfish.

CCK was initially discovered in the mammalian small intestine (Iinuma, Shibata et al. 2019) as an anorexigenic peptide secreted by the GI tract. CCK and two CCK receptors genes, CCK-1r and CCK-2r, have been isolated in goldfish (Tinoco, Valenciano et al. 2015). The CCK-1r receptor protein is widely distributed within the GI tract, while the CCK-2r receptor protein is mostly present in the brain (Rønnestad, Gomes et al. 2017). In goldfish, both intraperitoneal (IP) injections and intracerebroventricular (ICV) injections of CCK result in a decrease in feeding (Mandic and Volkoff 2018), highlighting the role of this peptide as anorexigenic in goldfish.

GLP-1 is a hormone released from the GI tract following food consumption. It stimulates the glucose-induced secretion of insulin and increases insulin expression (Donnelly 2012). In goldfish, GLP-1 IP injection decreased food intake (Blanco, Bertucci et al. 2017), and therefore is considered an anorexigenic peptide in this fish species.

PYY is a member of the neuropeptide Y (NPY) family, which includes NPY and pancreatic polypeptide (PP). However, unlike NPY, which stimulates appetite, PYY inhibits food intake. In goldfish, PYY mRNA expression has been found in the brain as well as the GI tract (Gonzalez and Unniappan 2010) and IP and ICV injections of PYY result in a decrease in food intake (Gonzalez and Unniappan 2010). Peptide YY is therefore, considered an anorexigenic peptide.

Recent evidence in mammals and fish suggests that the gut microbiota is involved in regulating digestive, metabolic, and appetite/ingestion processes, as well as the immune response (Burokas, Moloney et al. 2015, Gonçalves and Gallardo-Escárate 2017, Butt and Volkoff 2019) . The communication between the gut and the brain is referred to as the gut-brain-axis. The gut microbiota can influence this axis though interactions with both the gut and the brain, directly or indirectly, a pathway known as the microbiota-gut-brain axis. Several structures and systems are involved in the microbiota-gut-brain axis, including the neuroendocrine and neuroimmune systems, the central (CNS) and autonomic (ANS) nervous system, and the enteric nervous system (neurons within the GI tract) (Burokas, Moloney et al. 2015). The gut microbiota produces molecules/metabolites that interact with the enteric neurons of the host directly or indirectly, and ultimately influence GI motility and function (Yang and Chiu 2017). Direct mechanisms involve bacterial molecules within the gut lumen crossing the intestinal epithelial barrier to access enteric sensory neurons (Yang and Chiu 2017). Gut microbiota metabolites such as short chain fatty acids (SCFAs) or indoles are recognized by specific receptors (e.g. G-protein coupled receptors), on the surface of enteroendocrine cells of the GI tract, and can alter their absorptive and secretory capacity (Cani and Knauf 2016), therefore indirectly influencing appetite/digestive regulation. Most of the studies on gut microbiota have focused on mammals. However fish, who make up nearly half of the vertebrate species, have been studied to a far less extent (Tarnecki, Burgos et al. 2017). As the gut microbiota are known to play a key role in the overall health of the host, understanding the interactions between the microbiota and the fish host is an important area of research for the welfare of fish and aquaculture (Dehler, Secombes et al. 2017). This study will provide new insights into the effect of diet on the fish gut microbiota and subsequent regulation of genes related to appetite and digestion.

The objectives of this study were to (i) analyze the composition of the goldfish microbiota in various regions of the gut, i.e. foregut, midgut, and hindgut; (ii) examine the effects of feeding status (i.e. fed vs. fasted fish) on the composition of gut microbiota; (iii) examine the effects of synbiotics (live bacteria) addition to diets on the composition of gut microbiota and food intake as well as on the expression of genes related to appetite/digestion (OX, CART, CCK, GLP-1, PYY) to determine if changes in expression coincide with changes in microbial composition.

It is hypothesized the goldfish gut microbiota will differ among gut regions and the feeding status and diet will alter the goldfish gut microbiota as well as feeding behaviour and the expression of genes related to appetite and digestion.

2.3 Material and Methods

2.3.1 Experimental animals

Juvenile goldfish (*Carassius auratus*) (average length 9.042 ± 0.1612 cm; average weight 11.52 ± 0.3837 g) of both male and female sex were purchased from MSR Imporium Importations Canada (Lasalle, QC, Canada). Fish were housed in 65L tanks, with a constant flow of aerated and filtered water at 18°C, and fed once a day with Omega One Goldfish Flakes (39% protein, 11% fat, 2% fibre, 8.5% moisture), unless otherwise stated. Fish were acclimated under these standard conditions for two weeks prior to experiments. Fish of similar weight and length were selected for experimentation, and weighed (body weight) and measured (total length) at the end of the experiments. All fish were sacrificed by immersion in an overdose of MS-222 (TMS, tricaine methanesulfonate, Syndel Laboratories, Vancouver, BC, Canada), followed by spinal section. The fish brain and gut were dissected with gut regions separated into the foregut,

midgut, hindgut, and stored separately in 500ul of RNAlater (Qiagen, Mississauga, Ontario, Canada) at -20°C until further use.

All experiments followed the animal care protocols approved by Memorial University of Newfoundland Animal Care Committee following the guidelines of the Canadian Council on Animal Care guide to the care and use of experimental animals.

2.3.2 Food consumption

Food consumption was assessed in fish fed a control and synbiotic diet. Fish were fed once daily (12:00 noon) until apparent satiation, which was determined as the fish stopped searching and consuming food. The total weight of food given to each tank was recorded by measuring food before and after given by hand to the tanks. Feeding was performed in several rounds until fish reached satiety. Food consumption was measured as average daily food intake (g food/g fish) per tank and calculated by dividing the average amount of food consumed by the average weight of fish and the number of fish per tank.

2.3.3 Effects of nutritional status (i.e. fed and fasted, control and synbiotic diet)

To assess the effects of fasting on goldfish, fish were separated into eight 20L tanks, with four fish per tank for a total of thirty-two fish. Replicate tanks were present for all treatments to control for any tank effects. The experiment was conducted for a time frame of two and four weeks, with two tanks containing fish fed a commercial control diet of Nutrafin Bug Bites (45% protein, 12.5% fat, 5% fiber, 10% moisture) once a day for two weeks, another two tanks fasted for two weeks, and two tanks with fish fed the control diet once a day for four weeks, and two tanks fasted for four weeks (Table 2.1).

To examine the effects of synbiotics, fish were separated into eight 20L tanks with four fish per tank for total of thirty-two fish. Again, the experiment was conducted for a time frame of

two and four weeks, with two tanks containing fish fed a commercial control diet of Nutrafin Bug Bites (45% protein, 12.5% fat, 5% fiber, 10% moisture) once a day for two weeks, two tanks fed a diet of Dennerle Goldy Booster (54.7% protein, 11% fat, 3.2% fiber, 11.4% crude ash 8.4% moisture, including the prebiotic *Spirulina platensis* in composition and supplemented with the probiotic *Pediococcus acidilactici* CNCM MA 18/5M at $1x10^9$ CFU/g) for two weeks, and two tanks fed the control Nutrafin Bug Bites diet once a day for four weeks, and two tanks fed the Dennerle Goldy Booster for four weeks (Table 2.2). Food intake was quantified daily, as described above.

2.3.4 DNA Extractions

To perform microbiome analyses, DNA extractions were completed for the foregut, midgut, and hindgut regions of two goldfish under control conditions (fed Nutrafin Bug Bites for two weeks), as well as for the midgut of goldfish fed a control and a synbiotic diet, (probiotic/prebiotic experiment) and for fed and fasted fish (fasting experiment). All DNA extractions were performed using the QIAmp PowerFecal DNA kit (Qiagen) following the manufacturer's protocol. Total DNA quantity, purity and integrity were assessed for all DNA extracted samples using Nanodrop ND UV spectrophotometry (with 2ul used per individual DNA sample and acceptable concentrations set above 3 ng/µl).

2.3.5 Ion torrent amplicon sequencing

The Next Generation Sequencing (NGS) Ion torrent amplicon sequencing technology was used for sequencing of the V6-V8 region of the 16s rRNA in the foregut, midgut, and hindgut of two fish samples. This method utilized emulsion polymerase chain reaction (PCR) to replicate DNA sequences (Ramirez and Romero 2017), and is conducted on a bead surface within tiny water bubbles floating on an oil solution.

To prepare for sequencing, bar-coded primers were designed following the Ion Amplicon Library Preparation (Fusion Method). This method uses fusion primers to attach the Ion A and truncated P1 (trP1) adapters to the amplicons as they are generated in PCR. The primer design requires four fusion primers (two pairs of forward and reverse primers per target region) for bidirectional sequencing. This method allows for the target region to be simultaneously obtained on both strands of the DNA. However, the method was altered and bidirectional sequencing was not completed. Instead the target region of the DNA was generated separately for the forward and reverse regions, with only one A primer, linked to the forward primer, and one P primer linked to the reverse primer (one pair of forward and reverse primers per target region, Figure 2.1). Bar codes were selected from the Ion Xpress Barcodes 96.

Once the bar-coded primers were designed, samples underwent PCR with primers. A 25 µl reaction volume was produced with 12.5 µl of GoTaq, 0.5 µl of forward and reverse primer, 1 µl of DNA template, and 10.5 µl of water. The reaction mix was subjected to the following cycling program: 30 cycles 1) polymerase activation (94˚C for 5 minutes), 2) denaturation (94˚C for 1 minute), 3) annealing (49˚C for 30 seconds), 4) extension (72˚C for 2 minutes).

Agencourt AMpure Bead PCR purification was completed for DNA size selection following the manufacturer's protocol. This resulted in a DNA library with DNA fragments flanked by ion torrent adapters.

The Qubit dsDNA HS (High Sensitivity) Assay Kit was used to prepare DNA for quantification with the Qubit Fluorometer. This measures the DNA concentration in ng/ml. Samples were diluted to between 5 and 500 pg/ul to prepare for analysis with the bioanlayzer.

The Agilent 2100 Bioanalyzer was used for the sizing, quantification, and quality control of DNA samples before downstream analysis. The Agilent High Sensitivity DNA kit was used and the protocol provided by the guide was followed.

Following successful analysis with the Bioanlyzer, emulsion PCR was completed using the Ion OneTouch 2 Instrument following the Ion PGM ™ Hi-Q ™ View OT2 kit protocol (Ion Torrent). This method is a variation of PCR used to replicate DNA sequences before sequencing is done. It is conducted on a bead surface with tiny water bubbles floating on an oil solution. The result is beads with amplified sequences, which are placed on a chip and sequenced.

DNA sequences produced were edited in Geneious Prime 2019.2.1. This consisted of barcode splitting, primer trimming, low quality trimming, removal of short reads, and chimera removal. Following editing of sequences in Geneious, analysis of the 16S rRNA libraries with Qiime™ 1 (*qiime.or*g/) was conducted. Briefly, this consisted of preparing the library file in which a meta file was produced containing required information to identify samples. Next FASTA files were combined to create a file containing all the sequences from the FASTA files specified in the meta file. Taxonomy labels were then assigned using UCLUST (*omictools.com/uclust-tool*) and SILVA 97 (*www.arb-silva.de*) packages. Lastly, diversity graphs were created in which a summary table with all required information about the analysis was created along with bar-graphs, area-charts, and heat-maps.

2.3.6 Illumina sequencing and analysis

Midgut DNA samples for control and synbiotic as well as fed and fasted treated fish were sent to Laval University for sequencing. This method was used instead of Ion Torrent Amplicon Sequencing due to time constraints. Sequencing included preparation of 16S rRNA amplicons,

V6-V8 region, and MiSeq sequencing of amplicon pool using the Illumina TruSeq HT indexing strategy.

DNA sequences produced were then run on the SPONS-2 (Streamlined Process Of Next-gen Sequences, Version 2) pipeline as described in (Verhoeven, Salvo et al. 2018) to complete quality control, read-pair merging, operational taxonomic unit (OTU) creation and taxonomy assignment. The resulting OTU table and abundances were then used in R, for further downstream analysis and visualization, again, following the same workflow described in (Verhoeven, Salvo et al. 2018). Briefly, initial filtering was completed to remove of several OTUs with low prevalence. The remaining data set was then used in downstream analysis. A rarefaction curve was created to determine if sufficient sequencing depth was present for each sample. Alpha diversity (bacterial community richness) was determined by calculating the Hill's series of diversity indices for each sample using the square-root transformed count data. H_0 is analogous to the total number of species, H_1 is analogous to the exponent of Shannon diversity or "number of typical species", and H_2 is analogous to the reciprocal of the Simpson's index or "number of highly abundant species" (Verhoeven, Salvo et al. 2018). The beta-diversity analysis was used to assess differences in the microbial composition between treatments. The *count zero multiplicative method* was used in R and transformed with a *centered log ratio* (clr) transform and visualized with a PCA plot (Verhoeven, Salvo et al. 2018). In the fasting experiment, Aldex2 (ANOVA-Like Differential Expression) analysis was used to identify specific OTUs that are differentially abundant across fed and fasted treatment groups and two-week fasted and four-week fasted treatments. Effect size was used to establish significant differences instead of p-value across conditions, as p-values may give more false positives whereas effect size is stricter. Effect size was filtered to show only those values between < -1 or > 1. When comparing between

fed and fasted groups, a positive (+) effect number indicates a bacterial taxon which strongly correlates with the fed group, and a negative (-) effect number indicating a bacterial taxon which strongly correlates with the fasted group. For two-week fasted and four-week fasted group a positive effect number indicates a bacterial taxon which strongly correlates with the two-week fasted group, and a negative effect number indicates a bacterial taxon which strongly correlates with the four-week fasted group.

2.3.7 RNA extractions and cDNA synthesis

RNA extractions were completed for foregut and brain samples of goldfish administered a control and synbiotic based diet, as well as those under fed and fasted treatments. Total RNA was isolated using the *GeneJET*™ RNA Purification Kit (Fermentas, Burlington, ON, Canada) following the manufacturer's protocol. Final RNA quantity, purity and integrity were assessed for samples using A260/280 NanoDrop ND UV spectrophotometry (with 2 ul used per individual RNA sample, and acceptable ratios set at 1.8-2.0 for A260/280).

Reverse transcription (RT) was completed for RNA samples prior to qPCR. Isolated RNA was diluted to a concentration of 1 ug/ul and prepared for reverse transcription using iScript™ (Bio-Rad) or Verso (Thermo Fisher Scientific™) cDNA Synthesis Kits following the manufacturer's protocols. For iScript™ cDNA synthesis, 4.8 µl of iScript RT Supermix, 13.2 µl of nuclease free water, and 5 µl of RNA template were mixed for a total volume of 18 µl per reaction. The iScript RT Supermix includes a blend of oligo(dT) and random primers. For Verso cDNA synthesis, 4 µl of cDNA synthesis buffer, 2 µl of dNTP mix, 1 µl of RNA primer (oligo(dT) or random), 1 µl RT enhancer, 1 µl of Verso Enzyme mix, and 1 µl of RNA template were mixed for a total volume of 20 µl per reaction. The resulting cDNA samples were diluted and stored at -20˚C until further use.

2.3.8 Real-time quantitative PCR (qPCR)

To examine the effects of nutritional status on the expression of genes related to appetite and digestion, real-time quantitative PCR (qPCR) was completed. Foregut and whole brain RNAs were isolated from goldfish for synbiotic-treated, control, as well as fed and fasted fish as described above. Specific primer pairs (Tables 2.3, 2.4) were optimized for qPCR using serial dilutions of cDNA. Efficiency, R^2 values, and optimal annealing temperatures were determined for all primer pairs. Only primers with sufficiently high efficiency (90-110%) and linearity (R^2) of expression levels (R^2>0.99) were used. In addition, a melting curve analysis was performed at the end of each qPCR to verify primer specificity. Duplicate reactions were prepared using a reaction mix containing 5 µl 2x SensiFAST™ SYBR® No-ROX mix (Bioline, London, UK), 0.2 µl water, 0.4 µl 10 µM of forward primer, 0.4 µl 10 µM of reverse primer, and 4 µl of cDNA (diluted with 1:3 water). The total reaction volume (10 µl) was then loaded into the 96-well plate. The loaded plate was then placed in the CFX96 Touch™ Real-Time PCR Detection System (BioRad) and subjected to the following cycling program: 40 cycles; 1) polymerase activation (95˚C for 2 minutes), 2) denaturation (95 ˚C for 5 seconds), 3) annealing (60˚C for 10 minutes), 4) extension (72 ˚C for 20 seconds). Several reference genes were tested to determine the optimal candidate including actin, 18S rRNA, and elongation factor 1- (EF). Using NormFinder Software (*https://moma.dk/normfinder-software*), it was determined that actin was the most stable reference gene across all treatment groups, with the exception of the synbiotic and control foregut cDNA samples for which the 18S rRNA reference gene was the most stable. Gene expression levels were measured and quantified using CFX Maestro software (Biorad). The software compares expression levels using the ΔΔCt method. All samples are expressed as ratios of specific target gene to the reference gene and normalized as a percentage of mRNA

levels of fish in the control (either the control diet-fed fish at two weeks in the synbiotic experiment or the fed fish at two weeks for the fasting experiment) groups (set at 100%).

2.3.9 Statistics

Statistical analyses were completed in Prism 8 GraphPad Instat (Graphpad Software Inc., San Diego, California, USA). Data were tested for normality using the Shapiro-Wilk normality test. For food consumption, a parametric ANOVA followed by Tukey's tests was used to compare the control and synbiotic-fed groups. For gene expression, statistical differences between groups were assessed using either parametric ANOVAs followed by Tukey's tests or non-parametric Kluskal-Wallis tests followed by Dunn's multiple comparison tests. In some cases, Student's t-tests were conducted to compare two groups. Significance was set at $p < 0.05$.

Table 2.1: Fasting experiment tank set-up for goldfish, showing numbers of fish in each experimental tank.

Tank	**Treatment**	**# of fish**
1	2 Week Fed	4
2	2 Week Fed	4
3	2 Week Fasted	3
4	2 Week Fasted	3
5	4 Week Fed	4
6	4 Week Fed	4
7	4 Week Fasted	4
8	4 Week Fasted	4

*****Tanks 3 and 4 had one casualty each during the experiment. Both deaths happened within several days of the experiment beginning.

Table 2.2: Tank set-up used for the synbiotic and food consumption experiments for goldfish, showing numbers of fish in each experimental tank.

Tank	**Treatment**	**# of fish**
1	2 Week Control	4
2	2 Week Control	4
3	2 Week Synbiotic	4
4	2 Week Synbiotic	4
5	4 Week Control	4
6	4 Week Control	4
7	4 Week Synbiotic	4
8	4 Week Synbiotic	4

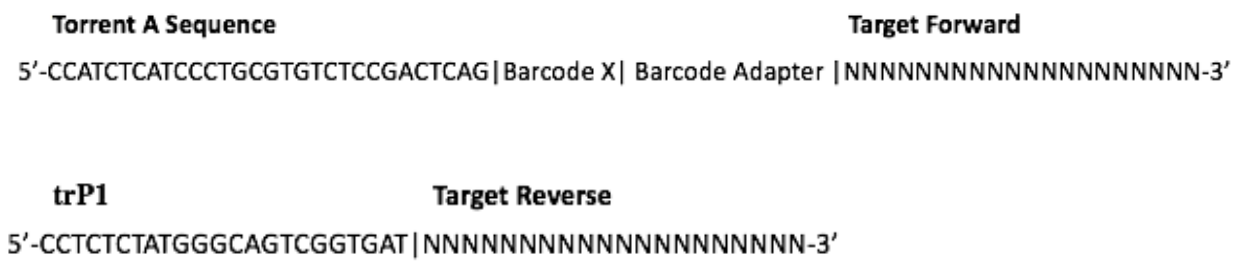


Figure 2.1: Barcode Fusion PCR primer design for DNA sequencing. The Barcode X and Barcode Adapter were selected from Ion Xpress Barcodes 96.

Table 2.3: Goldfish primers used for qPCR analysis of brain samples in synbiotic and fasting experiments with corresponding GenBank Accession ID (ACT: actin; CART1/2: cocaine- and amphetamine-regulated transcript; OX: orexin).

Target Gene	Sequence Name	Orientation	Primer Sequence (5' to 3')	GeneBank ID
ACT	gf act qF	Forward	ACTACTGGTATTGTGATGGACTCC	XM_026258408.1
	gf act qR	Reverse	CGGTCAGGATCTTCATCAGGTAG	
CART 1	gf CT1 qF	Forward	GTGCCGAGATGGACTTTGAC	AF28810.1
	gf CT1 qR	Reverse	AGCTGCTTCTCGTTGGTCAG	
CART 2	gf CT2 qF	Forward	GGAAAAGCTGCAGACGAAC	LN591117
	gf CT2 qR	Reverse	CGATTCGAGAGCCTTTTCTG	
OX	gf OF qF	Forward	GAGTTCAGCTGCTCCTCTTCA	XM_014270270.2
	gf OF qR	Reverse	ACTGCCGCGTCGTTATTAAA	

Table 2.4: Goldfish primers used for qPCR analysis of foregut samples in synbiotic and fasting experiments with corresponding GenBank Accession ID (CCK: cholecystokinin; GLP-1: Glucagon-like peptide-1; PYY: peptide tyrosine tyrosine).

Target Gene	Sequence Name	Orientation	Primer Sequence (5' to 3')	GeneBank ID
18S rRNA	gf 18S qF	Forward	AAACGGCTACCACATCCAAG	MN17056
	gf 18S qR	Reverse	CACCAGATTTGCCCTCCA	
ACT	gf act qF	Forward	ACTACTGGTATTGTGATGGACTCC	XM_026258408.1
	gf act qR	Reverse	CGGTCAGGATCTTCATCAGGTAG	
CCK	gf CCK qF	Forward	AACGCTGGAATCTGTGTGTG	XM_026284167.1
	gf CCK qR	Reverse	GGGGCTTCATCATCCTCT	
GLP-1	gf glcg qF	Forward	GCCTGGCTAAAATCCGGACA	XM_026264657.1
	gf glcg qR	Reverse	CGTGATGAAGCAGTCAGCAG	
PYY	gf PYY qF	Forward	CGGAGGATGTGATGGCTGAG	XM_026200184.1
	gf PYY qR	Reverse	GGAGGTTTTCTGTGGGAGGC	

2.4 Results

2.4.1 Microbiome composition in goldfish

2.4.1.1 Gut regions: foregut, midgut, hindgut

The phylum Firmicutes was predominant in all gut regions for both fish samples followed by phyla Proteobacteria and Fusobacteria (Table 2.5, Figure 2.2). There were no significant differences between similar gut regions of different fish samples (Figure 2.3), or between gut regions within the same fish sample (Figure 2.4).

Table 2.5: Goldfish gut microbiome composition at the phylum level in various regions of the gut. Data are represented as percentages of bacterial abundance (%).

	Fish 1			Fish 2		
	Foregut	Midgut	Hindgut	Foregut	Midgut	Hindgut
Taxonomy	%					
Bacteria;Other	0.1	0.1	0.1	0.1	0.1	0.1
Bacteria;_Acidobacteria	0	0	0	0	0	0
Bacteria;_Actinobacteria	0	0.1	0.3	0.3	0	0.1
Bacteria;_Bacteroidetes	0.8	0.1	0	0	0.2	0
Bacteria;_CK-1C4-19	0	0.1	0	0	0.5	0.3
Bacteria;_Chloroflexi	0	0	0	0	0	0
Bacteria;_Cyanobacteria	0	0.3	0	0	0	0
Bacteria;_Elusimicrobia	0	0	0	0	0	0
Bacteria;_Firmicutes	68.4	77.3	90	84.2	72.5	87.1
Bacteria;_Fusobacteria	12.3	9.6	0	0.5	6.4	0.7
Bacteria;_Proteobacteria	14	9.3	8	12.3	15.6	9.4
Bacteria;_Synergistetes	0	0	0	0	0	0
Unassigned;Other	4.4	3	1.6	2.5	4.7	2.3

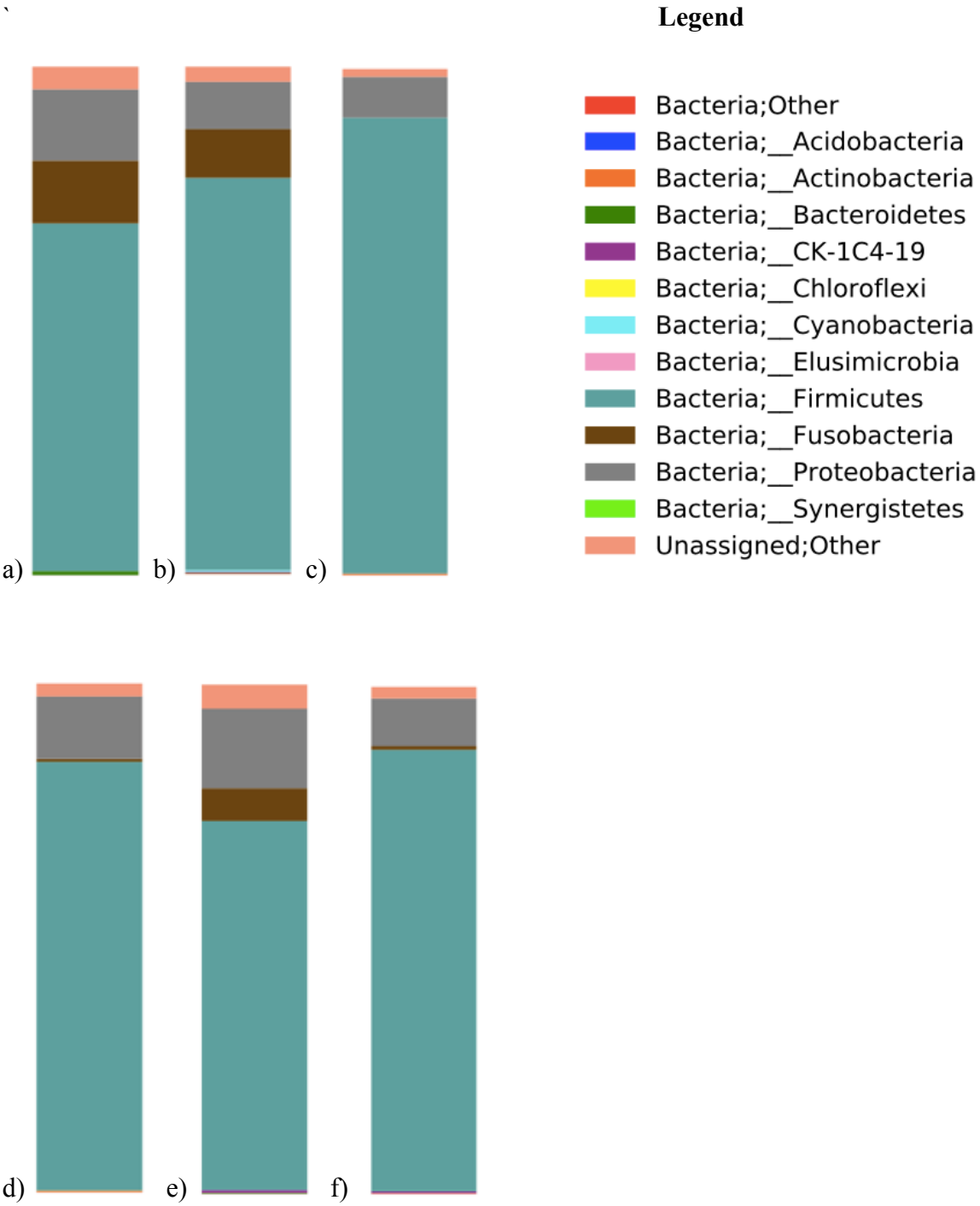


Figure 2.2: Goldfish gut microbiome composition at the phylum level in various regions of the gut (a) fish 1 foregut (b) fish 1 midgut (c) fish 1 hindgut (d) fish 2 foregut (e) fish 2 midgut (f) fish 2 hindgut.

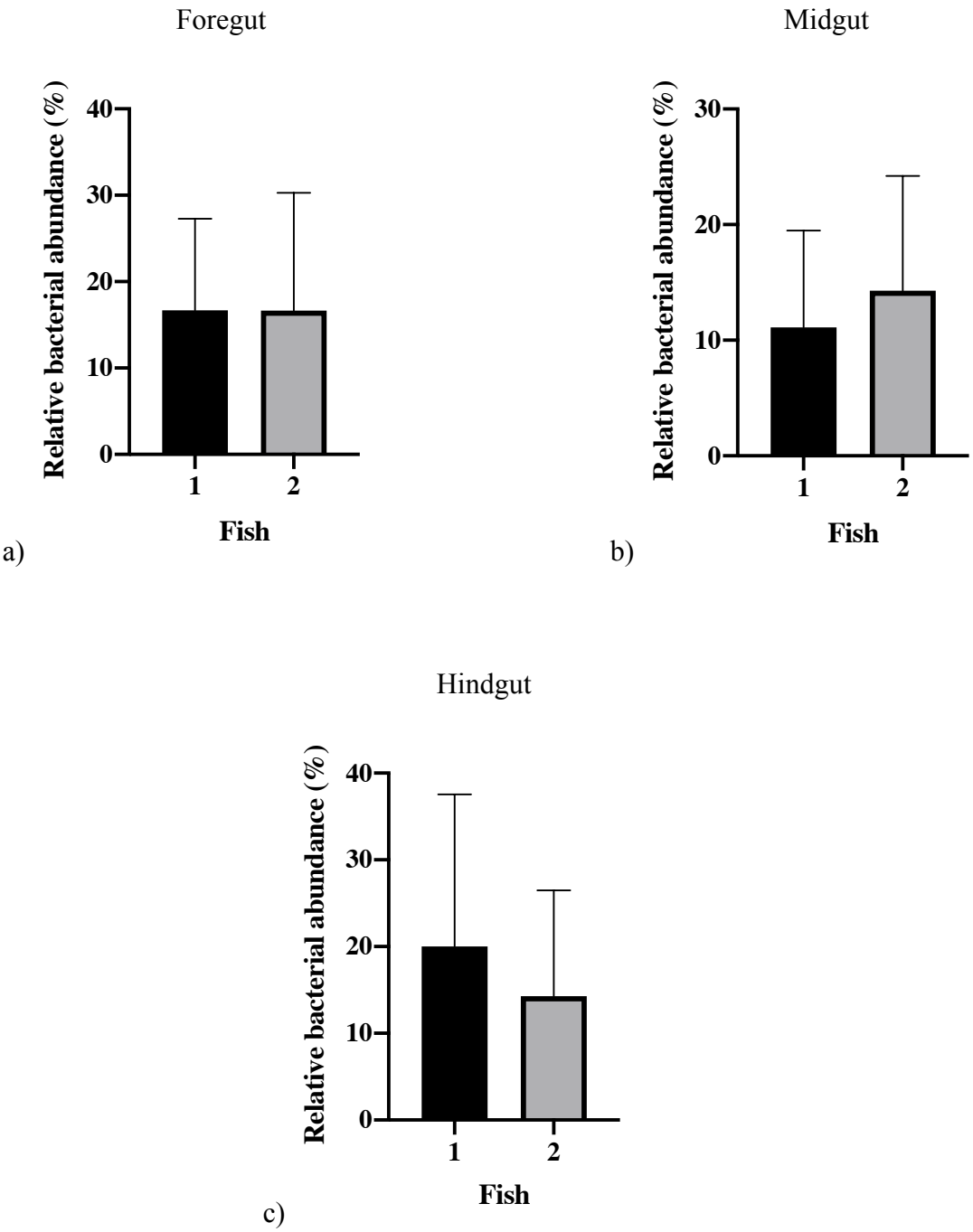


Figure 2.3: Goldfish relative bacterial phyla abundance for two fish samples varying gut regions. (a) Foregut, (b) Midgut, (c) Hindgut. Data are presented as % ± SEM.

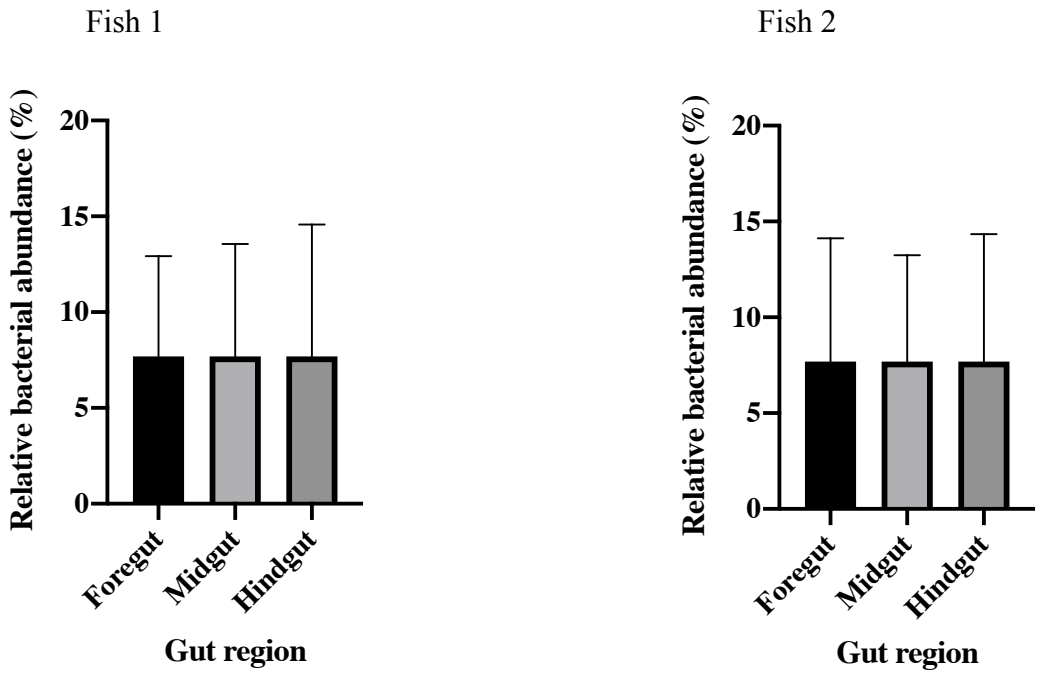


Figure 2.4: Goldfish relative bacterial phyla abundance among varying gut regions (Foregut, midgut and hindgut). (a) Fish 1, (b) Fish 2. Data are presented as % ± SEM.

2.4.1.2 Control/Synbiotic

2.4.1.2.1 Effects of synbiotic supplemented diets on food intake and the gut microbiota

There were no significant differences in food consumption for two-week synbiotic fed fish in comparison to the two-week control. However, fish administered a synbiotic diet for four weeks had a significantly higher food consumption compared to fish administered the control diet for four weeks (Figure 2.5). There was a significant increase in food intake between the control fish at two weeks and synbiotic fed fish at four weeks, and between synbiotic fed fish at two weeks and synbiotic fed fish at four weeks, as well as between the control fish at four weeks and synbiotic fed fish at four weeks.

Alpha diversity (bacterial community richness) was lower in the synbiotic fed fish than the control fish (hill number 0; number of species, t-test, $p<0.05$) but similar between the two and four week treatments (Figure 2.6). Variations in the taxonomic composition at the phylum, order and genus levels were present among individual samples, as indicated by the variation in abundance of taxonomic groups (Figure 2.7 a, b, c, d). The phylum Bacteriodetes was predominant in all samples (Figure 2.7 a). The majority of control and synbiotic samples were dominated by order Aeromonadales and genus *Aeromonas* (Figure 2.7 b, c).

Beta diversity was similar between control and synbiotic samples (ADONIS, $p=0.973$) but differences were seen between the two-week and four week samples (ADONIS, $p=0.001$) (Figure 2.8).

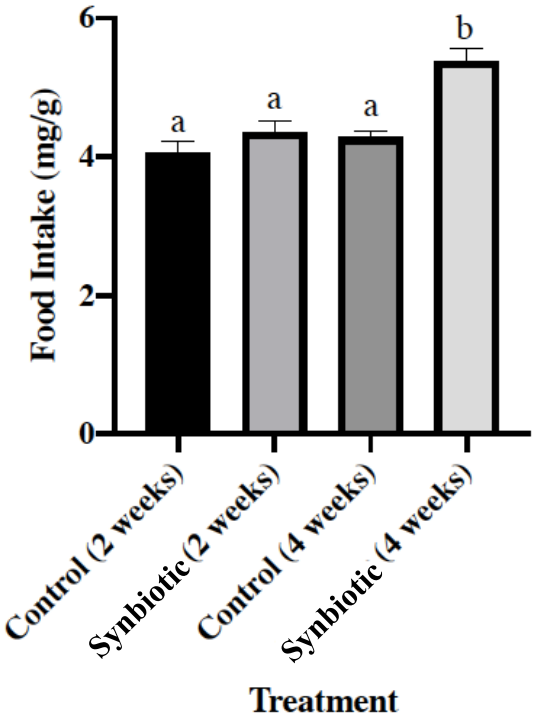


Figure 2.5: Effects of diet composition on food intake. Data are mean ± SEM, n= 8 fish per group. Bars with dissimilar superscripts indicate groups that differ significantly.

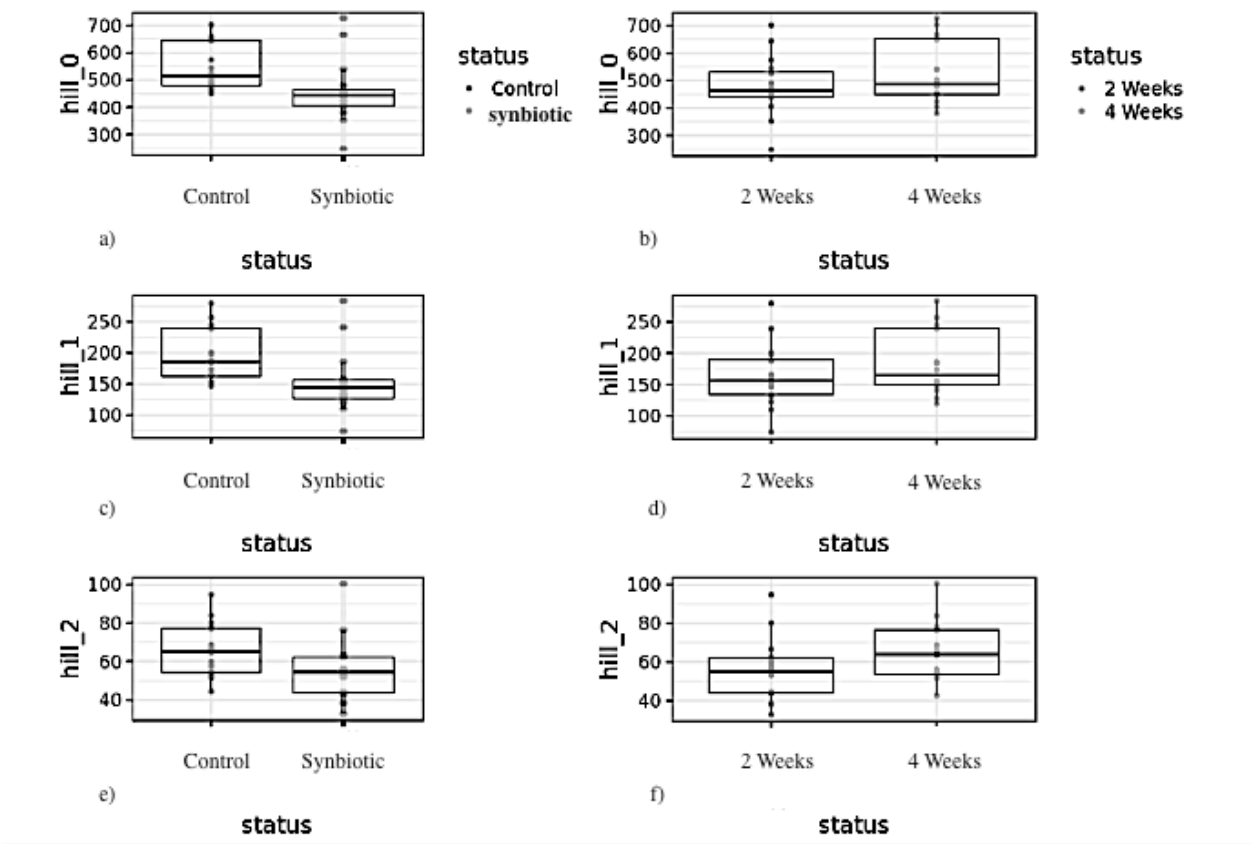


Figure 2.6: Alpha diversity (community richness) in fed and fasted and control and synbiotic fed fish. H_0 is the total number of species, H_1 is the exponent of Shannon diversity ("number of typical species"), and H_2 is the reciprocal of the Simpson's index ("number of highly abundant species"). (a) H_0 control and synbiotic, (b) H_0 2 and 4 weeks, (c) H_1 control and synbiotic, (d) H_1 2 and 4 weeks, (e) $Hill_2$ control and synbiotic, (f) $Hill_2$ 2 and 4 weeks.

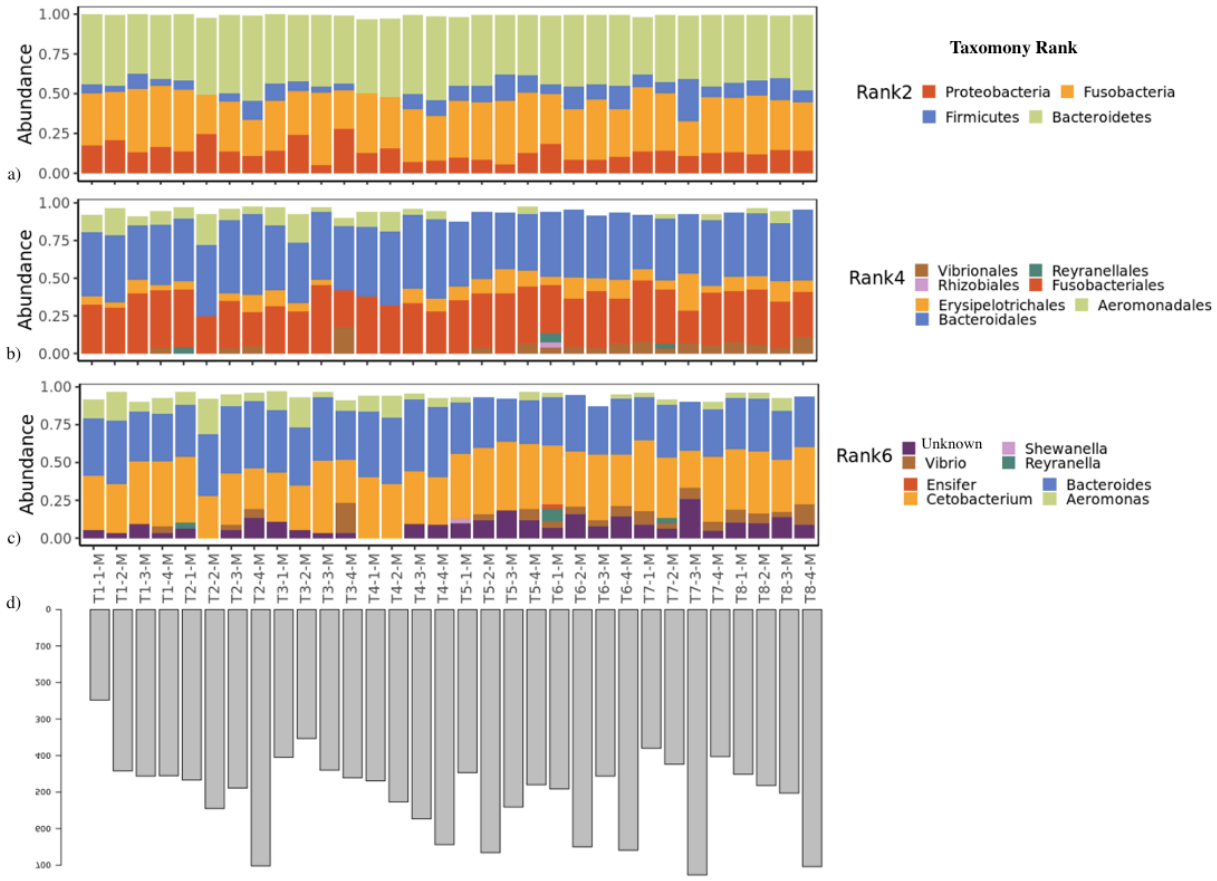


Figure 2.7: Overview of the taxonomic composition per-sample in control fish (labels T2, T4, T6, T8) and synbiotic fed fish (labels T1, T3, T5, and T7). a) Taxonomic composition at the phylum level b) Taxonomic composition at the order level c) Taxonomic composition at the genus level d) Biodiversity as number of species H_0 detected in each sample.

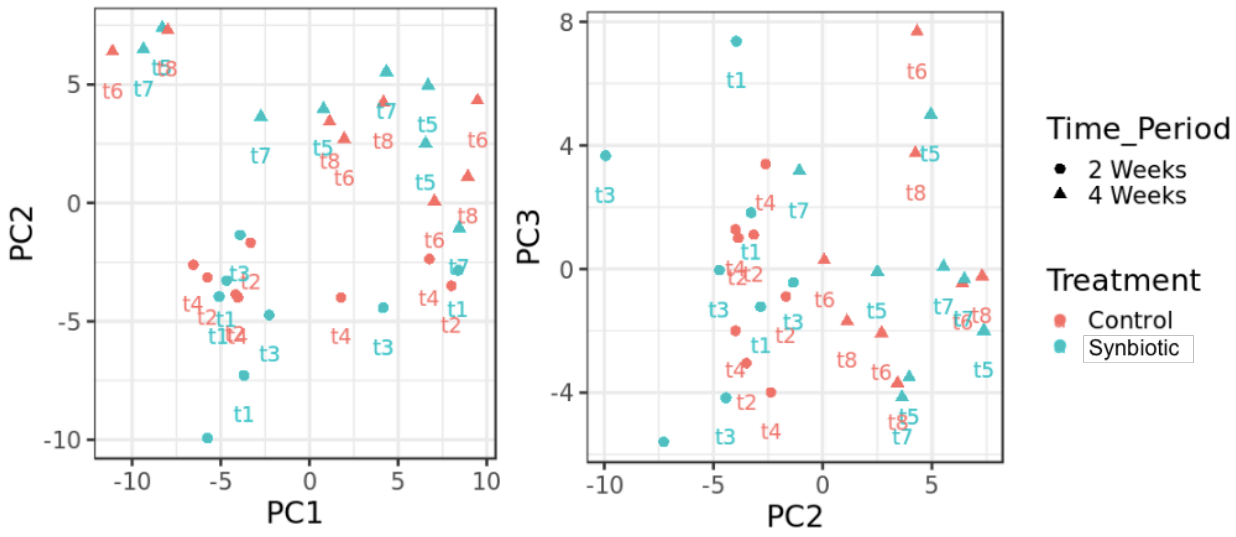


Figure 2.8: PCA plot of synbiotic and control samples. Samples are separated by distances signifying multivariable differences a) control and synbiotic, (ADONIS, p=0.973) b) 2 and 4 weeks, (ADONIS, p=0.001).

2.4.1.3 Fed/Fasted

The alpha diversity analyses show fed fish samples did not differ significantly from fasted fish samples at hill 0 (t-test, p value 0.9583), hill 1 (t-test, p value 0.7381), or hill 2 (t-test, p value 0.1513) (Figure 2.9). No effect of time period was detected (Figure 2.10): the two-week fed samples did not significantly differ from four-week fed samples at hill 0 (t-test, p value 0.4382), hill 1 (t-test, p value 0.3635), or hill 2 (t-test, p-value 0.1509). Likewise, no significant differences were found between two-week fasted samples and four-week fasted samples at hill 0 (t-test, p value 0.8588), hill 1 (t-test, p value 0.7183), or hill 2 (t-test, p value 0.7080).

The beta diversity analysis indicates time is not likely a factor on the fed treatment groups at two and four weeks. In the fasted treatments, differences were apparent between the two and four weeks groups, but these were not significant (Figure 2.11).

The Aldex2 analysis indicated several significant differences in abundance of OTUs between fed and fasted groups (Table 2.6) and two and four-week fasted groups (Table 2.7). The absolute differences between the fed and fasted groups was up to 10.14 (Table 2.6), and -10.9 (Table 2.7). The genera *Shewanella* and *Aeromonas* were correlated with the fasted group, whereas *Vibrio, Bacteroides, Cetobacterium, Vibrio, and Reyranella* were correlated with the fed group (Table 2.6). The genera *Flavobacterium* and *Aeromonas* were correlated with the four week fasted group and *Bacteroides* were correlated with the two week fasted group (Table 2.7).

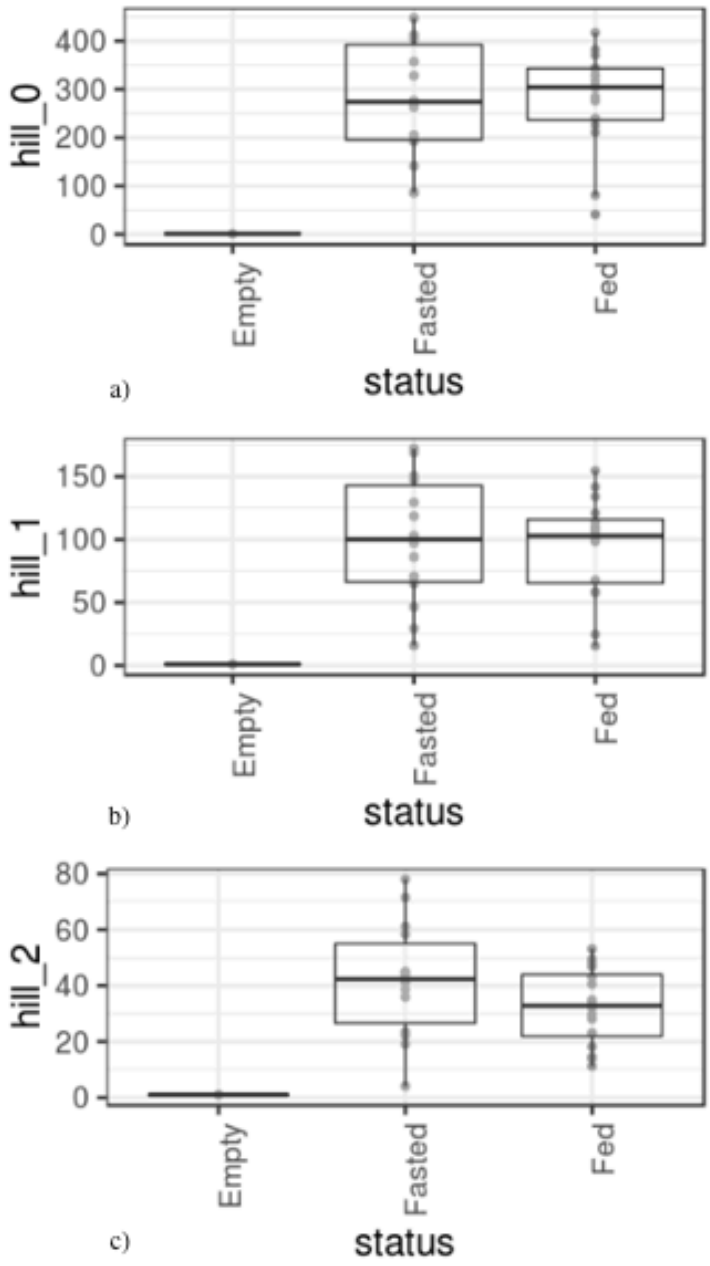


Figure 2.9: Alpha diversity to determine community richness. Hill_0 is the total number of species, hill_1 is the exponent of Shannon diversity ("number of typical species"), and hill_2 is the reciprocal of the Simpson's index ("number of highly abundant species"). (a) hill_0 fed and fasted, (b) hill_1 fed and fasted (c) hill_2 fed and fasted.

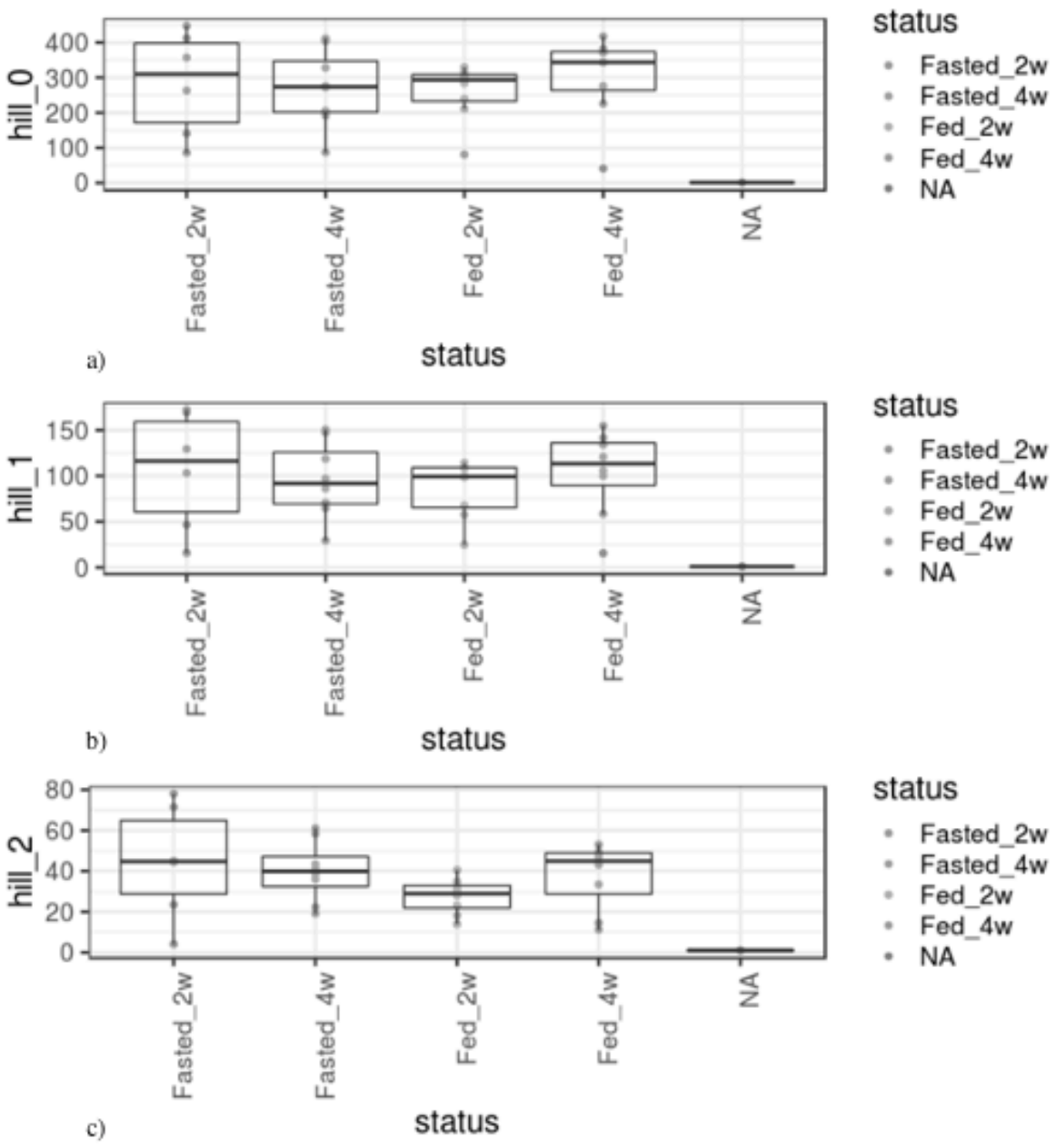


Figure 2.10: Alpha diversity to determine community richness. H_0 is the total number of species, H_1 is the exponent of Shannon diversity ("number of typical species"), and H_2 is the reciprocal of the Simpson's index ("number of highly abundant species"). (a) Hill_0 2 weeks fasted, 4 weeks fasted, 2 weeks fed, 4 weeks fed, (b) Hill_1 2 weeks fasted, 4 weeks fasted, 2 weeks fed, 4 weeks fed (c) Hill_2 2 weeks fasted, 4 weeks fasted, 2 weeks fed, 4 weeks fed.

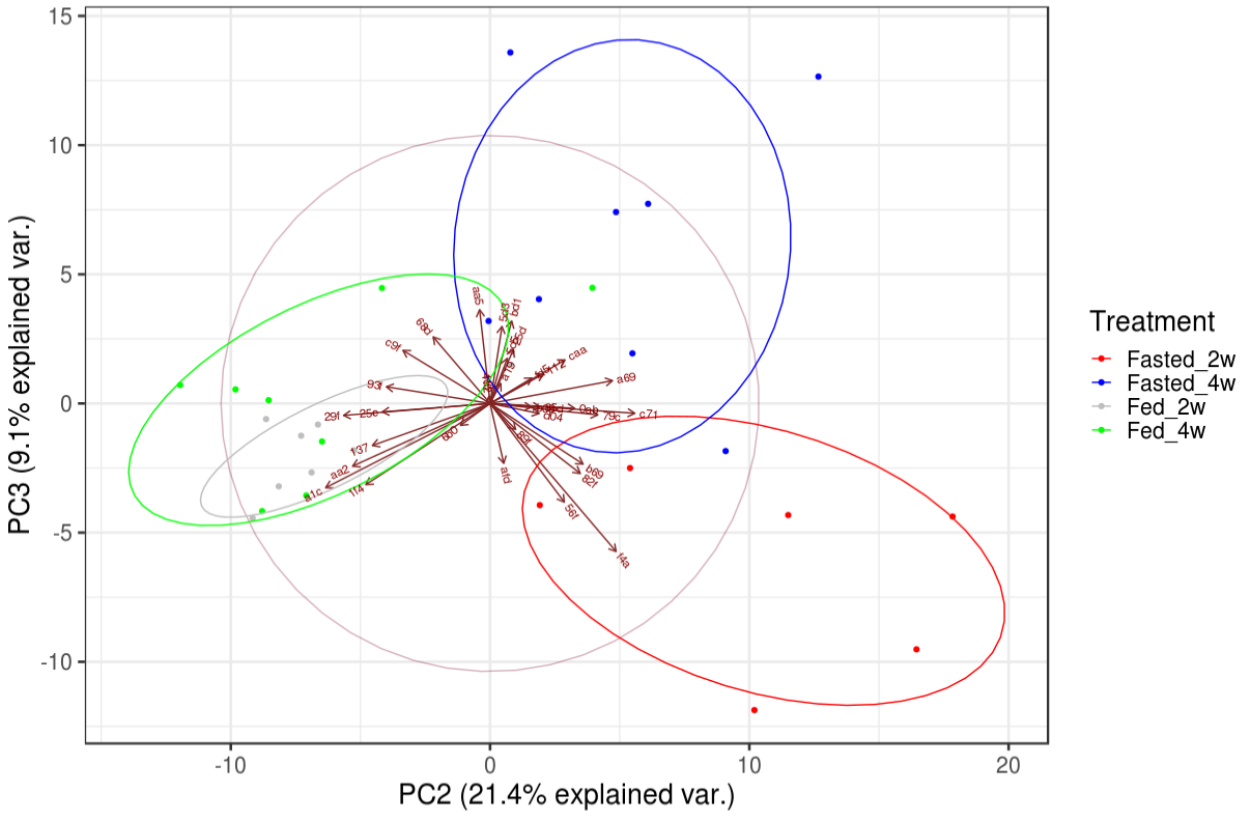


Figure 2.11: CLR transformation for beta diversity for 2 weeks fasted, 4 weeks fasted, 2 weeks fed and 4 weeks fed.

Table 2.6: List of significantly different taxa for fed vs. fasted treatment groups.

Taxonmy Rank								
Phylum	**Class**	**Order**	**Family**	**Genus**	**Species**	**diff.btw**	**diff.win**	**effect**
Proteobacteria	Gammaproteobacteria	Vibrionales	Vibrionaceae	Vibrio	NA	5.153648	4.435278	1.103955
Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides	NA	4.95635	4.076616	1.1033
Proteobacteria	Gammaproteobacteria	Alteromonadales	Shewanellaceae	Shewanella	morhuae	-5.0612	4.234104	-1.09852
Proteobacteria	Gammaproteobacteria	Aeromonadales	Aeromonadaceae	Aeromonas	allosaccharophila	7.275705	4.702944	1.378715
Proteobacteria	Gammaproteobacteria	Aeromonadales	Aeromonadaceae	Aeromonas	allosaccharophila	7.150047	5.934178	1.003972
Fusobacteria	Fusobacteriia	Fusobacteriales	Fusobacteriaceae	Cetobacterium	NA	3.165628	2.878338	1.143303
Proteobacteria	Gammaproteobacteria	Vibrionales	Vibrionaceae	Vibrio	NA	10.14573	7.161426	1.256929
Proteobacteria	Gammaproteobacteria	Alteromonadales	Shewanellaceae	Shewanella	xiamenensis	-7.26343	5.433109	-1.20609
Proteobacteria	Alphaproteobacteria	Reyranellales	Reyranellaceae	Reyranella	NA	7.948197	6.172842	1.106909
Proteobacteria	Gammaproteobacteria	Aeromonadales	Aeromonadaceae	Aeromonas	caviae	-7.87675	6.319323	-1.07776
Fusobacteria	Fusobacteriia	Fusobacteriales	Fusobacteriaceae	Cetobacterium	NA	6.184991	4.729969	1.172643
Proteobacteria	Gammaproteobacteria	Vibrionales	Vibrionaceae	Vibrio	NA	5.622063	4.292413	1.196915
Proteobacteria	Alphaproteobacteria	Reyranellales	Reyranellaceae	Reyranella	NA	4.495163	3.554158	1.164669
Proteobacteria	Gammaproteobacteria	Aeromonadales	Aeromonadaceae	Aeromonas	dhakensis	-7.5276	5.329163	-1.34059

Note: Where difference between (diff.btw), median difference in centered log ratio (clr) values between fed and fasted groups; difference within (diff.win), largest median variation within fed group, or fasted group; effect, effect size of the difference median of diff.btw/diff.win; overlap, proportion of effect size that overlaps 0 (i.e. no effect), and NA indicates the taxonomy rank could not be identified.

Table 2.7: List of significantly different taxa for two-week fasted vs. four-week fasted groups.

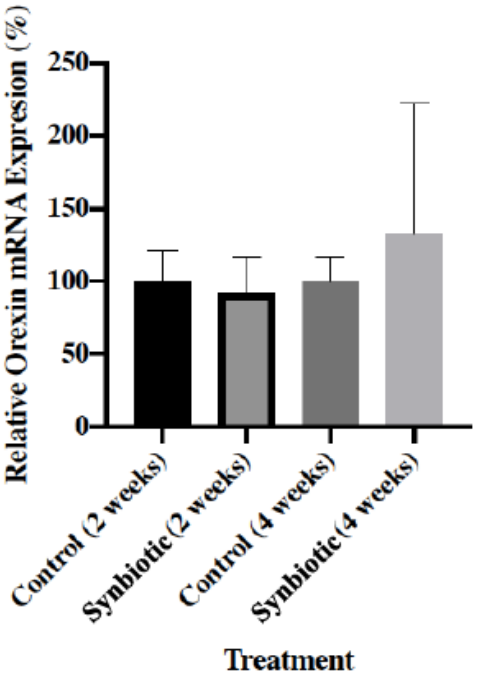
Taxonmy Rank								
Phylum	**Class**	**Order**	**Family**	**Genus**	**Species**	**diff.btw**	**diff.win**	**effect**
Bacteroidetes	Bacteroidia	Bacteroidales	Barnesiellaceae	NA	NA	8.288806	6.948962	1.062958
Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides	NA	7.169523	6.94639	1.003212
Bacteroidetes	Bacteroidia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	cucumis	-7.32763	6.697616	-1.19248
Proteobacteria	Gammaproteobacteria	Aeromonadales	Aeromonadaceae	Aeromonas	NA	-10.9068	8.658988	-1.30712

Note: Where difference between (diff.btw), median difference in centered log ratio (clr) values between 2-week fasted and 4-week fasted groups; difference within (diff.win), largest median variation within 2-week fasted group, or 4-week fasted group; effect, effect size of the difference median of diff.btw/diff.win; overlap, proportion of effect size that overlaps 0 (i.e. no effect), and NA indicates the taxonomy rank could not be identified.

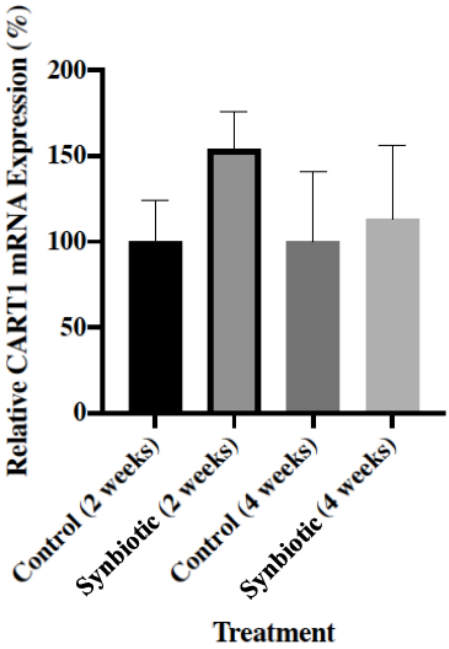
2.4.2 Effects of nutritional status on the expression of genes related to appetite and digestion

2.4.2.1 Control/Synbiotic

There were no significant differences in relative brain mRNA expressions of orexin, CART1 and CART2 at the two and four-week periods between control and synbiotic-fed goldfish (ANOVA, Figure 2.13). The mRNA expressions of CCK, and GLP-1 in the foregut did not differ between the control and synbiotic-fed fish at two and four weeks (ANOVA, Figure 2.14). When comparing only two-week control and two-week synbiotic, PYY expression was higher in the synbiotic group (Student t-test, $p<0.05$).



a)



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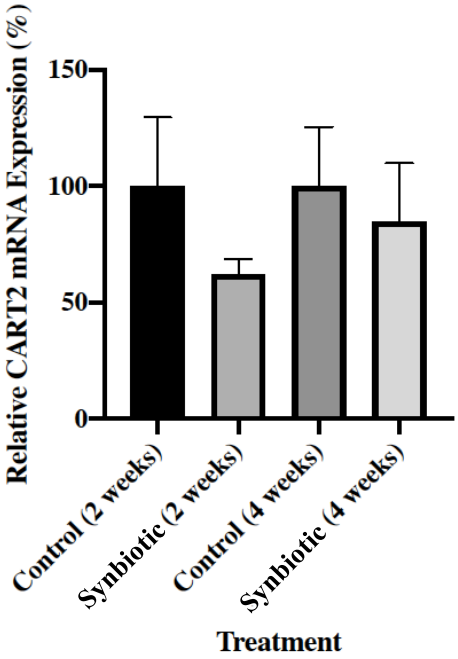
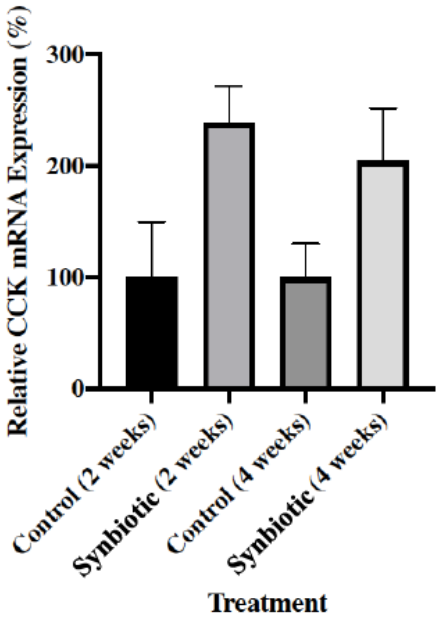
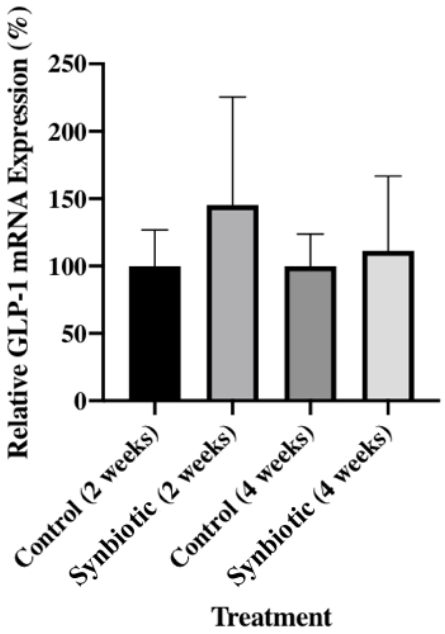


c)

Figure 2.13: Relative brain mRNA expression of a) orexin, b) CART1, and c) CART2 in control and synbiotic fed goldfish at two and four weeks (n = 2-6 per group). Data are represented as mean ± SEM.



a)



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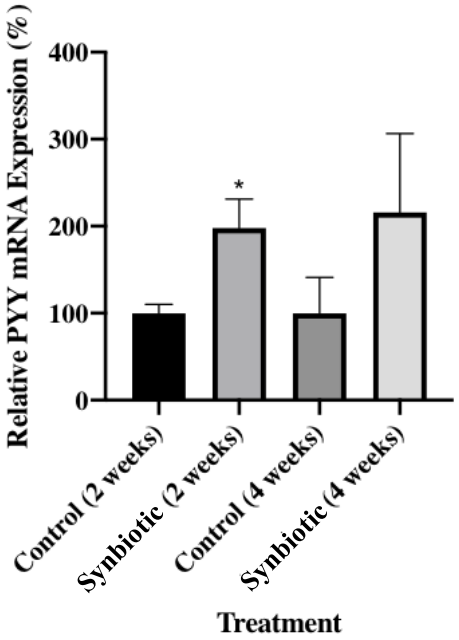
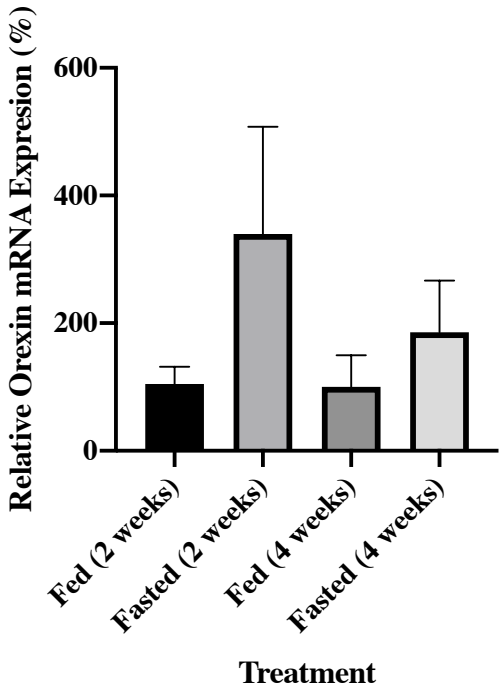


c)

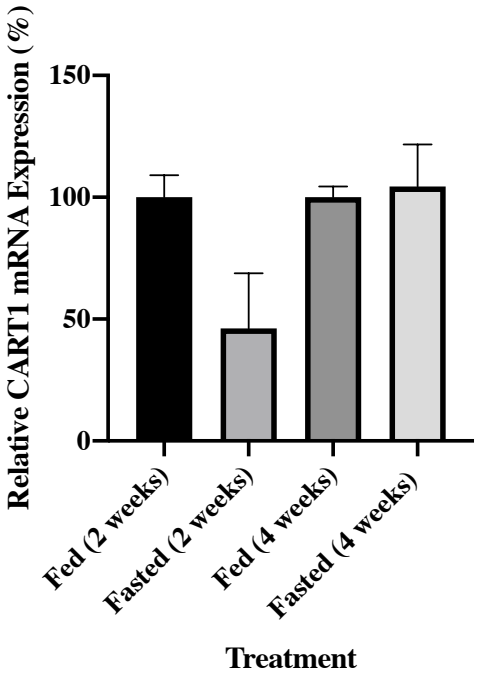
Figure 2.14: Relative foregut mRNA expression of a) CCK, b) GLP-1, and c) PYY in control and synbiotic fed goldfish at two and four weeks (n = 2-6 per group). Data are represented as mean ± SEM. The asterisk indicates significant differences between the control and synbiotic at a given time period (2 weeks or 4 weeks) (t-test, $p<0.05$).

2.4.2.2 Fed/Fasted

There were no significant differences in brain orexin and CART1 relative mRNA expressions between fed and fasted fish (Figure 2.15). CART 2 expression in the four weeks fasting fish was lower than in the two-week fasted. CART2 expression was lower in the four weeks fed fish than the two-week fasted (Figure 2.15). Fasting decreased in goldfish foregut CCK and PYY relative mRNA expressions for both the two and four-week time periods (Figure 2.16). GLP-1 expression was higher in the four-week fasted fish compared to the two-weeks fed, the two-weeks fasted and the four-weeks control fish (Figure 2.16).



a)



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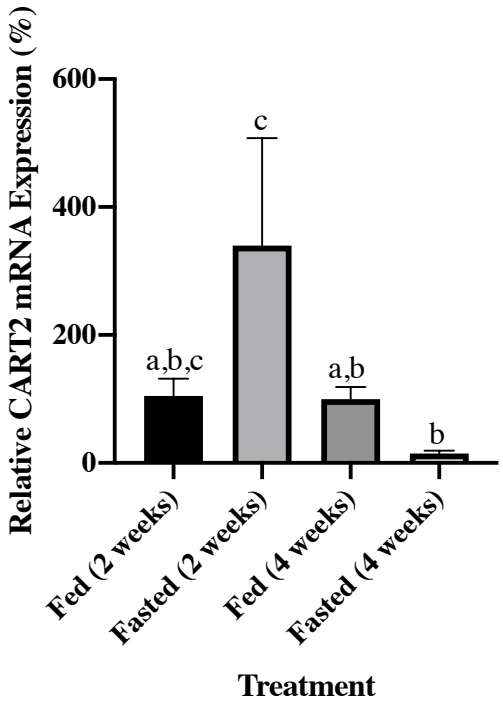
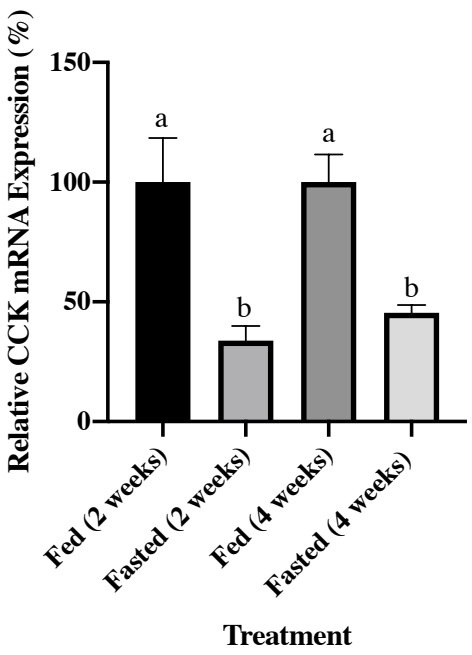


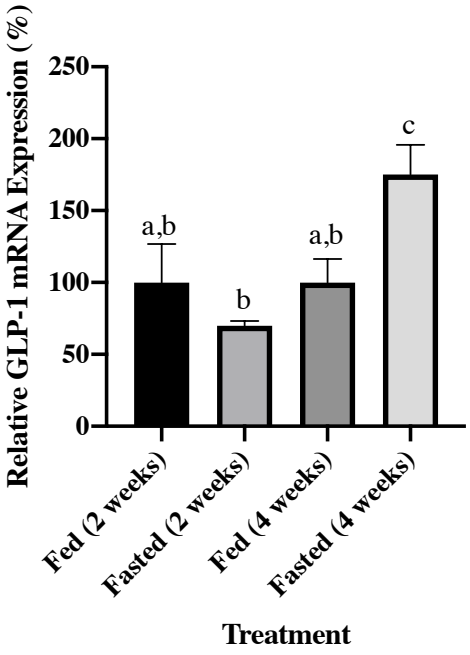
c)

Figure 2.15: Relative brain mRNA expression of a) orexin, b) CART1, and c) CART2 in control fed and fasted goldfish at two and four weeks (n = 2-6 per group). Data are represented as mean

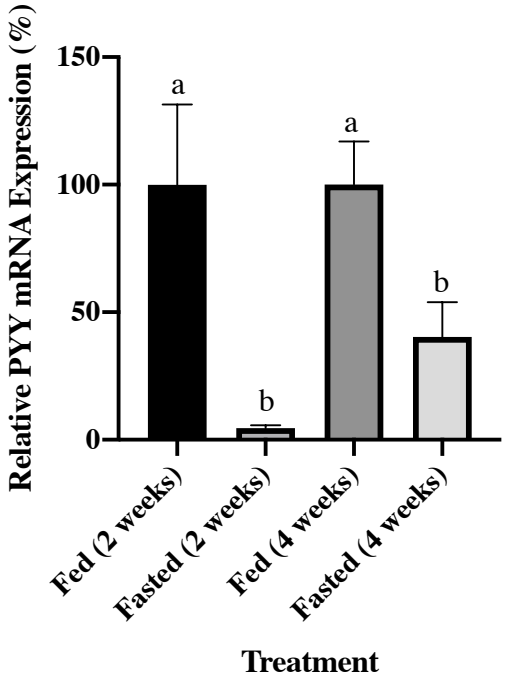
± SEM. Bars with dissimilar superscripts indicate groups that differ significantly. (t-test, $p<0.05$).



a)



b)



c)

F**igure 2.16:** Relative foregut mRNA expression of a) CCK, b) GLP-1, and c) PYY in control fed and fasted goldfish at two and four weeks (n = 2-6 per group). Data are represented as mean ± SEM. Bars with dissimilar superscripts indicate groups that differ significantly. (t-test, $p<0.05$).

2.5 Discussion

The goals of this study were to examine the goldfish gut microbiota, and determine if nutritional status would result in variations within the gut microbiota, and if these changes would coincide with changes in gene expression in the brain and gut. I first examined the gut microbiota in various regions of the goldfish gut, i.e. the foregut, the midgut, and hindgut to compare the microbiota composition between these regions. I then examined the gut microbiota of goldfish under varying diets such as control and synbiotic fed fish, as well as fed and fasting conditions to identify any effects of nutritional status on the gut microbiota. Lastly, I investigated the effects of nutritional status (control and synbiotic, fed and fasted) on mRNA expression levels of appetite regulators. I found a significant increase in PYY mRNA expression in the midgut for synbiotic-administered fish and a significant decrease in CART2, CCK, and PYY mRNA expressions in fasted fish.

2.5.1 Microbiome composition in goldfish

2.5.1.1 Gut regions: foregut, midgut, hindgut

The phylum Firmicutes was predominant in all gut regions for both fish samples examined followed by the phyla Proteobacteria and Fusobacteria. This was expected as Fusobacteria have been found to be predominant in freshwater fish (Zhang, Li et al. 2017) and several studies have reported the presence of the Firmicutes and Proteobacteria phyla in goldfish, including *Cetobacterium somerae* (phylum Firmicutes), *Aeromonas hydrophila*, *A. punctate*,

S*hewanella putrefaciens* as well as the *Pseudomonas* genus *Vibrio* genera and the family Enterobactriaceae (phylum Proteobacteria) (Sugita, Tsunohara et al. 1987, Asfie M 2003, Silva, Nicoli et al. 2011). Therefore, the gut microbiota composition found here does not vary from previous observations in goldfish.

No differences were seen within similar gut regions of different fish samples. These results are not surprising as fish were kept under the same control conditions and feeding regime. No differences were seen between gut regions within individual fish, suggesting that the microbiota is uniformly distributed along the intestine of goldfish. Our results differ from that of other studies using different species in which the gut microbiota composition varies depending on the intestinal regions considered. For example, in Southern catfish (*Silurus meridionalis*), differences in the dominating taxa are seen between the hindgut and foregut (Zhang, Li et al. 2017). It is noteworthy that my study was based on only two fish, and more variability in the microbiota might have been seen had more fish samples been examined.

Given the microbial stability among gut regions found in this study I chose the midgut for subsequent microbiota analyses and foregut for gene expression studies.

2.5.1.2 Control/Synbiotic

2.5.1.2.1 Effects of synbiotic supplemented diets on food intake and gut microbiota

Fish fed a synbiotic diet had higher food intake when compared to the fish fed control diet. An increase in food consumption following a synbiotic diet has previously been shown in other fish species such as Nile tilapia (Giorgia, Elia et al. 2018) and pacu (Cerozi 2012). In fact, synbiotics are commonly used in aquaculture to improve appetite, growth, and feed conversion (Newaj-Fyzul, Al-Harbi et al. 2014). However, other studies have shown no effect [e.g. tambaqui (de Azevedo, Fosse Filho et al. 2016)] or a decrease [e.g. zebrafish (Falcinelli, Rodiles et al.

2016); grouper (Wang, Yang et al. 2018)] in food intake following a probiotics diet, suggesting that the effect is dependent on the species and the probiotics examined. In my study, the increase in food intake was only seen after four weeks. It is possible that a longer exposure to the synbiotics diet was necessary for goldfish to "adapt" to the new diet and perhaps cause a shift in the microbiome that might have affected food intake. This is consistent with the fact that in rainbow trout, switching the fish from a fish meal to a soy bean meal induces changes in the gut microbiota after 18 weeks but not in the initial 8 weeks (Heikkinen, Vielma et al. 2006). Therefore, the time in which the synbiotic diet is administered can be suggested to influence the changes observed in the gut microbiota and subsequent feeding habits.

Feeding fish with a diet containing the probiotic bacterium *Pediococcus acidilactici, and* prebiotic, *Spirulina platensis*, caused a decrease in the biodiversity of the gut microbiota composition in goldfish in comparison to the fish fed a control diet, at both two and four weeks. This may be the result of the synbiotic diet increasing the presence of beneficial bacterial strains causing the overall diversity of the community to be lower. This has been shown in several fish species, in which probiotic administration increased the beneficial bacteria, rather than the microbial diversity richness. Similar to my results, in red tilapia (*Oreochromis niloticus*), administration of the same probiotic, *P. acidilactici*, decreases richness and diversity (Ferguson, Merrifield et al. 2010), a result of microbial community competition exerted by the supplemented probiotic, in which a number of species are removed (Ferguson, Merrifield et al. 2010). Similarly, in zebrafish larvae administered the probiotic *Lactobacillus rhamnosus*, the microbial diversity is significantly lower when compared to the control group (Falcinelli, Picchietti et al. 2015), suggesting that the probiotic strain might have increased the abundance of the Lactobacilli genera and reduced the presence of potential pathogens (Falcinelli, Picchietti et

al. 2015). Studies examining the effects of the prebiotic *Spirulina platensis* on the fish gut microbiota are limited. However, this prebiotic has been shown to promote the growth of *Lactobacillus acidophilus* in vitro (Bhowmik et al., 2009). Again, this may explain the decrease in microbiota diversity observed in fish administered synbiotic.

Analysis of the taxonomic composition revealed similarities between synbiotic and control fed fish gut microbiota. The phylum Bacteriodetes was predominant in all samples and the majority of control and synbiotic samples were dominated by the order Aeromonadales and genus *Aeromonas.* Interestingly, the administered probiotic, which belongs to the phylum Firmicutes, order Lactobacillales, and genus *Pediococcus,* was not predominant nor was the prebiotic belong to the phylum Cyanobacteria, order Spirulinales, and genus *Spirulina*. It is likely the four-week time period was not long enough to allow for the establishment of the *Pediococcus* or *Spirulina* in the gut. It is possible that the concentration of the synbiotic in the diet was not sufficient to make it detectable in the synbiotic-fed group.

Inter-sample fluctuations in the biodiversity could be seen, not only between samples within the same treatment group (synbiotic and control), but between samples of differing treatments. The variations in the microbiota composition among fish may be due to several factors. Although goldfish samples within the same treatment groups were exposed to the same rearing conditions and diet, variations may be present as a result of host-specific intrinsic factors including genetics and sex, as both male and female fish were examined. Variations between samples of different treatment groups were expected as these groups were exposed to different diets. The dietary ingredients, type of food, and food additives may exert effects on growth of certain fish gut microbes and greatly influence the structure and composition of the gut microbiota (Vatsos 2016). Therefore, variations seen among samples within the same treatment

group can be attributed to genetics, and variations seen among samples from different treatment groups can be attributed to the altered diet.

The beta diversity analysis indicated no clear differences in the species composition between synbiotic and control samples. This may indicate the resilience of the core gut microbiota associated with the goldfish as the taxonomic composition remained stable. This has been shown in rainbow trout for which the core gut microbiota identified contained taxa present regardless of diet (Terova, Rimoldi et al. 2019).

An effect of time period could be seen in the beta diversity analysis, as two and four-week data points were located on differing sides (left and right respectfully). This indicates the two and four-week groups differed in species composition. The differences between two and four week samples may be a result of goldfish adapting to the diet during the initial two weeks causing a shift in the microbiome from two to four weeks.

2.5.1.3 Fed/Fasted

There were no clear differences in the alpha diversity between fed and fasted fish following analysis at hill 0, 1, or 2 at either two or four-weeks, suggesting that the overall bacterial composition was not affected by fasting. However, beta diversity analysis highlights the effect of time period on the fed and fasted treatment groups. Although time is not likely an effect on the fed treatment groups at two and four weeks, differences are present among the fasted treatment groups at two and four weeks. This may be a result of the goldfish adjusting to the lack of food, causing the microbiome to shift and favour opportunistic bacterial taxons, similar to what was found in the control and synbiotic groups.

The taxa listed in Table 2.6 and Table 2.7 are indicative of the taxa that best explain the variation present among fed and fasted groups and two-week fasted and four-week fasted groups,

respectively. It is important to note that this method of analysis examines the changes in abundance of a taxon relative to all other taxons present in the data set (Gloor and Reid 2016). The taxon Proteobacteria Aeromonadales *Aeromonas allosaccharophila*, was strongly correlated with the fed group whereas the Proteobacteria Aeromonadales *Aeromonas aeromonasdhakensis*, was strongly correlated with the fasted group. It may not be surprising that *Aeromonas* species were both strongly correlated in both the fed and fasted groups, as these bacterial species have been classified in goldfish as the 'permanently indigenous type', which are not disturbed by the diet (Sugita, Tsunohara et al. 1988). The Gammaproteobacteria was the only taxonomic class present when defining the taxa which explain the variation among the fed and fasted groups. This may indicate the microbial shift favour opportunistic bacteria, as suggested previously. The *Vibrio* and *Cetobacterium* genera were correlated with the fed group. Species from these genera are suggested to be inactive, and unaffected by diet in goldfish (Silva, Nicoli et al. 2011). These results indicate the microbiota composition varies under fed and fasted conditions.

Two-week fasted group was dominated by the order Bacteroidales, whereas the four-week fasted group included both the Flavobacteriales and Aeromonadales orders. Once again, the presence of the *Aeromonas* genus in the four-week fasted group is not surprising as this is a genus not disturbed by diet. Bacteroidetes was present in the two and four week fasting groups and Proteobacteria was present in the four-week fasting group. These two phyla have been shown previously to be abundant and predominant in long-term fasting goldfish (Li, Qi et al. 2019). The largest effect size (1.063) for the two-week fasted and four-week fasted was seen for the phylum Bacteroidetes (genus *Bacteroidales*), indicating this taxon is greatly correlated with the two-week fasted group. Bacteroidetes has been shown to be particularly abundant in long term fasting in goldfish (Li, Qi et al. 2019). The taxon most strongly correlated (-1.307) with the

four-week fasted group was Proteobacteria Aeromonadales *Aeromonas*. Proteobacteria has been found to predominate long-term fasting goldfish (Li, Qi et al. 2019). These results indicate the period of time fasted influences the gut microbiota composition.

Although fasting is known to influence the physiology of fish (Day, Tibbetts et al. 2014), the response of the gut microbiota to fasting is still unclear. Early studies using culture-based methods found that fasting reduced bacterial density in fish (Margolis 1953). In tilapia, fasting induces an increase in the microbial phylogenetic diversity in the colon but a decrease in the phylogenetic diversity of the cecum (Kohl, Amaya et al. 2014), suggesting that changes in the gut microbiota may vary among gut regions. My results suggest that the goldfish foregut microbiota might be responsive to fasting.

2.5.2 Effects of nutritional status on the expression of genes related to appetite and digestion

2.5.2.1 Control/Synbiotic

There were no differences in the relative mRNA expression of brain orexin, CART1, and CART2 or foregut CCK, GLP-1, and PYY between the control and synbiotic fed fish at either two or four-weeks. It is possible that the synbiotic administration was not at a sufficient dosage or long enough to cause significant changes in expression. Indeed, synbiotics administered at various doses and different time periods might have different effects on relative gene expression of appetite regulators. For example, in goldfish, the administration of the probiotic *Lactobacillus acidophilus* at high, but not low doses, decreases intestine ghrelin gene expression and this effect is only seen after eight weeks (Hosseini, Kolangi Miandare et al. 2016). Furthermore, the type of administration could have affected the results, as different results have been obtained when administering probiotics as single or multiple bacterial species (Carnevali, Maradonna et al.

2017). This may be a result of the potential difference in likelihood of the probiotic to survive and colonize within the gut when administered as a single or multiple bacterial species.

Interestingly, in my study, the foregut mRNA expression of PYY exhibited a significant increase for the two-week synbiotic administered diet when a t-test was conducted ($p<0.05$). This may be a result of the synbiotic fed fish having a higher food intake when compared to the control fed fish, causing the expression of the anorexigenic PYY to increase. In fact, although no significant differences were found in other anorexigenic appetite regulators, there does seem to be a tendency for mRNA expression of these hormones to increase in the synbiotic fed goldfish. This might be due to the high variation in gut expression seen within groups or a too small number of samples analyzed.

2.5.2.2 Fed/Fasted

In my study, fasting did not affect the brain mRNA expressions of orexin or CART1. This is likely due to the outlier values indicated by the error bars, and the low number of samples analyzed. These results contrast with previous reports showing a fasting-induced increase in orexin expression (Abbott and Volkoff 2011) and decrease in CART1 expression (Volkoff 2001). CART2 expression decreased in the four weeks fasted compared to both the four weeks fed and the two weeks fasted, which is consistent with the fact that CART2, like CART1, is an anorexigenic hormone which expression has previously been shown to decrease during fasting in goldfish (Volkoff and Peter 2001). It is noteworthy that in my study, I examine expression in whole brain and it is possible that more pronounced changes would have been detected if specific brain regions (e.g. hypothalamus) had been used.

In the foregut, CCK and PYY both showed significant decreases in mRNA expression for both the two and four-week time periods. This is expected as both of these appetite regulators are

anorexigenic, and have previously been shown to decrease feeding (Gonzalez and Unniappan 2010, Mandic and Volkoff 2018). GLP-1 significantly increased in four-weeks fasted fish, as well as between two weeks fed and four weeks of fasting, and between the two-week fasted and four-week fasted groups. This is unexpected as GLP-1 is a anorexigenic peptide, and has been shown to decrease food intake in goldfish (Blanco, Bertucci et al. 2017). However, in some species such as the sea bass, GLP-1 has been shown to initially increase during short-term fasting followed by decrease (Cardoso, Felix et al. 2018). The initial increase in GLP-1 has been suggested to mediate the shift from carbohydrate metabolism to mobilization of energy stores.

2.6 Conclusion

Overall, my results suggest a link between the gut microbiota and appetite regulation in goldfish. Changes in the gut microbiota as a result of changes in the diet coincided with changes in the expression of genes related to appetite and digestion (Table 2.8).

The administration of a synbiotic diet for a two and four-week time period caused a decrease in gut microbiota biodiversity. This might be the result of the probiotic strain, *Pediococcus acidilactici,* and the prebiotic, *Spirulina platensis*, increasing the presence of beneficial bacterial strains causing the overall diversity of the community to be lower. Furthermore, analysis of the taxonomic composition revealed similarities in gut microbiota between synbiotic and control fed fish. However, slight variations are present in the abundance of taxonomic groups among individual samples within the same treatment group (synbiotic and control), as well as between samples of differing treatments. These differences in the goldfish gut microbiome due to the synbiotic diet may relate to changes in food consumption and expression of genes related to appetite and digestion. The synbiotic-fed goldfish had a higher food intake in comparison to the control fed fish when administered for a four-week period. As fish consuming

the synbiotic diet were eating more it is not surprising that there was a trend for an increase in anorexigenic in the foregut for the goldfish fed this diet for two weeks.

The microbiome of fed and fasted fish presented an effect of time in the fasted groups, but not in the control. In two-week and the four-week fasting periods differences were present in the species composition. Fasted goldfish had significant decreases in the expression of anorexigenic hormones (CART2 in the brain and CCK, and PYY in the foregut) when compared to the control fish, suggesting that appetite inhibiting peptides are decreased during fasting.

This study shows changes in the goldfish gut microbiota diversity and taxonomic composition coincided with changes in diet. Varying diets resulted in changes in food consumption and the regulation of appetite regulators. However, due to the complexity of the gut microbiota and appetite regulation in fish, as well as the need for standardizing microbiota studies, this study does not suggest direct causation. Therefore, continuing research is needed to fully explore this topic.

Table 2.8: Summary of Results

		Fasting		Synbiotic	
		2 weeks	**4 weeks**	**2 weeks**	**4 weeks**
Alpha Diversity		No effect	No effect	↓	↓
Beta		No effect	No effect	No effect	Sig Difference
Gene Expression	**Brain**	No effect	No effect	No effect	No effect
	Foregut	↓ CCK, PYY	↓ CCK, PYY ↑ GLP-1	↑ PYY	No effect
Food Intake		No applicable		No effect	↑

Chapter 3: General Conclusion

The gut microbiota plays a significant role in fish energy homeostasis and appetite regulation. There are a relatively limited number of studies which consider the manipulation of the gut microbiota via prebiotics, probiotics, synbiotics or fasting in fish and the subsequent effect on energy homeostasis and appetite regulation and the results are varied. This study contributes to current knowledge on this topic and may aid in current research involving goldfish, with the potential to extend to other cyprinids. Overall, I found for the first time in goldfish that (1) there are no major differences in microbiota composition across gut regions, in contrast to other previously studied fish species; (2) changes in the gut microbiota occur as a result of a change in diet and these coincide with changes in the regulation of appetite regulators (Orexin, CART1, CART2, CCK, GLP-1, PYY).

The synbiotic diet altered the gut microbiota by decreasing the biodiversity, possibly a result of the probiotic strain dominating and or increasing the predominance of select bacteria. In addition, goldfish fed the synbiotic diet had a higher food intake, which could be explained by the observed decrease in intestinal anorexigenic hormones such as PYY and a trend for the remaining hormones in the foregut to decrease (CCK, GLP-1). However, the administration of a synbiotic diet did not affect the expression of brain appetite regulators, which might indicate that synbiotic-diet induced changes in food intake are mediated mostly by peripheral signals.

Not surprisingly, overall, fasting decreased the expression of anorexigenic hormones (brain CART2 and gut CCK and PYY). Fasting affected the microbiome as abundance of OTUs differed between fed and fasted groups and two and four-week fasted groups. This suggests the goldfish gut microbiota might be responsive to fasting, and that these responses are time dependent.

Investigating the gut microbiota in fish as it relates to energy homeostasis and appetite regulation presents several challenges that should be considered when conducting further studies. First, the variety of methods used for studying the fish gut microbiota seen among studies makes the results difficult to compare and highlight the need for standardized methods. In addition, many factors affect the gut microbiota composition including environmental and genetic factors. Fish are very diverse with regards to habitats, and feeding habits and behaviours, making generalizing among species difficult, as major differences will likely be seen between species (for examples carnivores and herbivore, or tropical and cold-water species). It is thus crucial to study a variety of fish species in the future to fully understand the underlying mechanisms of the fish gut microbiota and energy homeostasis and appetite regulation. In addition, we only examined one combination of synbiotic. This synbiotic diet could be altered to contain an alternative combination of prebiotic and probiotic species to determine if varying synbiotics produce similar results.

The time frame for the administration of synbiotics is an important factor, as it has been shown to influence the gut microbiota composition, which may in turn influence the energy homeostasis and appetite regulation. In this study, the synbiotic diet was given for four weeks, which might not have been long enough to allow for the establishment of the prebiotic and probiotic in the gut. Further studies using a longer time frame of synbiotic administration should be conducted to assess the effects of time.

Finally, further insights on this topic could be obtained by examining the expression of brain and gut peptides which did not indicate significant differences between treatment groups by repeating the experiment with increasing sample sizes. Furthermore, addition brain and gut

peptides such neuropeptide Y (NPY) and ghrelin could be examined as the regulation of appetite involves a number of peptides, only some of which were investigated here.

There is a great potential in the fish model for studying the gut microbiota, and the use of probiotics as an alternative to antibiotics in aquaculture. This study shows an influence of diet, in particular a synbiotic diet, on the gut microbiota composition and the potential for the manipulated microbiome to regulate the expression of genes related to appetite and digestion and appetite behaviour to ultimately increase feeding and growth in fish.

The limitations in this study indicate the need for more research to fully explore this topic. Research is required to fully determine the underlying mechanisms; however, this study provides novel data showing a link between the gut microbiota composition and appetite regulation in goldfish.

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